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DOPA DECARBOXYLASE IN *DROSOPHILA MELANOGASTER*: AN ANALYSIS OF CROSS-REACTING MATERIAL AND ENZYME ACTIVITY IN NORMAL AND MUTANT STRAINS

by

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ABSTRACT

Dopa decarboxylase (DDC, E.C. 4.1.1.26) from *Drosophila melanogaster* was purified and antibodies elicited against DDC were used to measure the amount of immunologically cross-reacting material (CRM) present in mutant strains. The mutant strains belonged to two different classes. The first designated as *l(2) amd*, were recessive lethal on standard food and dominant lethals on food containing α-methyl dopa (AMD). AMD is a substrate analog and noncompetitive inhibitor of DDC. The second class of mutants, designated as *l(2) Ddc* were also recessive lethals and had reduced levels of DDC.

The *l(2) amd* mutants, as heterozygotes, did not exhibit any substantial differences in the activity or CRM levels of DDC. The *l(2) Ddc* mutants, as heterozygotes, had 28-55% of the enzyme activity and 43-75% CRM of the control. The *l(2) Ddc* mutants could be classified into three distinct classes based on their activity and CRM levels. The class I mutants behaved like true null mutants and apparently produced no DDC polypeptide. Both class II and class III mutants apparently produced mutant DDC polypeptides. The mutant polypeptides of class II mutants were more defective than the mutant polypeptides of class III mutants. The severity of mutant defects, as judged from the activity and CRM levels observed, agrees with the intragenic complementation pattern exhibited by *l(2) Ddc* mutants.

In an attempt to identify putative regulatory mutants, 27 wild type stocks were screened for altered levels of DDC. All these stocks were isogenic for a different second chromosome. One strain, BI-140, was

found to have 25% more activity than the Canton Special strain. The observation that it also had 20% more CRM than normal possibly makes it a strain containing a mutant in a regulatory locus affecting DDC.

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This thesis is dedicated to my parents

INTRODUCTION

The regulation of gene expression in eukaryotes is presently explained in terms of a differential regulation of gene activity at different times and in different tissues during development. The actual mechanisms involved are for the most part unknown, but attempts to understand the mechanisms constitute a very active area for modern molecular biologists and geneticists.

Dopa decarboxylase (DDC, E.C. 4.1.1.26) decarboxylates dopa (3,4 dihydroxyphenylalanine) to dopamine (3,4 dihydroxyphenylethylamine) and is found in both the hypodermis and neural tissues. In *Dipterans* the enzyme appears to have three functions. It was observed in the mosquito, *Aedes aegypti*, that there was an increase in DDC levels after the adult females were given a blood meal. The blood meal was necessary for the initiation of ovarian development and DDC caused a darkening and hardening of newly deposited eggs (Schlaeger and Fuchs, 1974a,b). DDC also has a role in the production of catecholamines, including dopamine, which serve as neurotransmitters in insects (Dewhurst *et al.*, 1972). Thirdly, DDC plays an important role in the sclerotization and tanning of larval and adult cuticles in insects. Extensive work has been done on this aspect of the function of DDC in *Calliphora erythrocephala*.

DDC is located in the epidermal cells of *Calliphora* and an increase in activity was observed as the developing larvae underwent cuticular sclerotization at the larval molts, puparium formation, and eclosion of the adult (Karlson and Sekeris, 1966a, Sekeris and Karlson, 1966). The actual sclerotizing agent does not appear to be dopamine but rather an acetylated derivative, N-acetyl dopamine (Sekeris and Karlson, 1962).

Karlson and coworkers also showed that the appearance of DDC activity at the time of puparium formation was induced by the steroid molting hormone, ecdysone. This was further confirmed by Shaaya and Sekeris (1965) in *Calliphora* and Chen and Hodgetts (1974) in *Sarcophaga bullata*. Fragoulis and Sekeris (1975a,b) have shown that the increase in DDC activity induced by ecdysone is due to the translation of newly synthesized mRNA molecules. The mRNAs were isolated from epidermis of *Calliphora* and when incubated in a ribosomal system *in vitro* under conditions of protein synthesis, produced DDC, which was identified by immunoprecipitation with specific antiserum against DDC.

Two peaks of ecdysone are found in *Bombyx mori* (Shaaya and Karlson, 1965), in *Calliphora* (Shaaya and Sekeris, 1965) and in *Drosophila* (Hodgetts *et al.*, 1977); these occur at puparium formation and later just prior to the secretion of the adult cuticle. Residual levels of ecdysone are known to be present in the adult stages of several insects (Hodgetts *et al.*, 1977, King, 1970)and are known to play a role in oogenesis in insects (Hagedorn *et al.*, 1975; Lagueux *et al.*, 1977; Legay *et al.*, 1976). DDC in *Drosophila* also shows two peaks of activity (Lunan and Mitchell, 1969; McCaman *et al.*, 1972) which occur at puparium formation and eclosion. The peak of DDC activity at eclosion appears well after the second peak in ecdysone titer. Thus, there seems to be more than one regulatory control in this system; the DDC activity is being induced by ecdysone at puparium formation, at a later stage ecdysone has no effect on the DDC activity and still later DDC activity is seen in the absence of ecdysone. The second peak of DDC activity could be independent of ecdysone. Alternatively, synthesis of DDC mRNA, induced during the second ecdysone

peak could result in a "masked" product not translated until just prior to adult eclosion.

Not much is known about the mechanisms of ecdysone action. In the 1960's Karlson and his coworkers (Karlson, 1965, 1966, 1968; Karlson and Sekeris, 1966b) suggested that the hormone might act as an effector molecule and combine with repressor proteins, thus allowing certain genes to be transcribed. They compared it to the Jacob and Monod scheme of bacterial enzyme induction (Jacob and Monod, 1961a,b). Later in the early 1970's, Emmerich (Emmerich, 1970a,b) presented evidence which showed that ecdysone reversibly binds to proteins in the cytoplasm and is transported into the nucleus where he postulated it could function as an effector molecule. He also observed that ecdysone bound to the acidic chromatin proteins and not to the nucleohistones. These observations compare well with those on progesterone, a vertebrate steroid hormone. Spelsberg *et al.* (1971, 1972) have shown that progesterone binds to a receptor protein, the oviduct cytosol protein and this hormone-receptor complex binds ten times more effectively to the chromatin than does the hormone alone. Cytosol protein from non-target tissues did form a hormone-receptor complex but did not bind to the oviduct chromatin. A specific fraction of acidic proteins, *viz* the AP_3 fraction, associated with the chromatin was seen to enhance the binding of hormone receptor complex to the oviduct chromatin. This system exhibits a positive control mechanism where hormone-receptor complex enhances the binding to the target tissue chromatin. The model proposed by Karlson and coworkers suggests a negative control system wherein the hormone combines with the repressor protein, thus enabling certain genes to be transcribed.

Yund and Fristrom (1975) have studied the uptake and binding of ecdysone in imaginal discs of *Drosophila melanogaster*. They observed that uptake and retention of ecdysone caused an increase in RNA synthesis. Two types of hormone binding sites were recognized--nonspecific sites not subject to competition and specific sites subject to competition, which were saturated at the low hormone concentrations which induce morphogenesis. Results obtained in experiments using inhibitors like actinomycin D, cycloheximide, and N-ethylmalemide suggested that the binding sites already existed in the cell prior to an exposure to the hormone and that functional sulphydryl groups were needed for binding.

Studies of the generalized effects of the hormone such as those carried out by Yund and Fristrom will not lead to a complete understanding of the mechanism of ecdysone action. This will require an analysis of the specific effects of ecdysone on the expression of a particular gene. It is our hope that the study of DDC will provide a useful model system for this purpose.

The genetic approach to the problem has been to induce and recover mutations in *Drosophila melanogaster* that affect the levels of DDC. Initially it was thought that some could be regulatory mutants and some could be mutants in the structural gene. Wright and coworkers employed a structural analog of dopa, α-methyl dopa (AMD), as an inhibitor of DDC to screen for mutants with abnormally high and low levels of DDC activity in flies. AMD added to standard *Drosophila* media is lethal to the developing organisms and times of death correspond with periods at which DDC activity are known to peak. Mutants with elevated levels of DDC should be resistant to a normally lethal concentration of AMD and mutants which decrease DDC activity should be sensitive to normally

tolerable concentrations.

Sherald and Wright (1972, 1974) isolated three strains which showed greater resistance to AMD than the wild type. Two of these strains showed significantly higher levels of DDC activity (20-40%). One of these strains was isolated directly by high DDC activity and was later found to be resistant to AMD. There was no indication that DDC had been altered in these mutants as seen by thermolability studies and *in vitro* response to AMD. The second chromosome was found to be associated with both high DDC activity and resistance to AMD induced lethality. The left arm of the second chromosome was responsible for the increase in DDC activity in these strains while both the left and right arms of the second chromosome contributed to the increase in resistance. It thus appeared that screening for resistance to AMD had some potential as a method for recovering mutants which produced excess amounts of DDC.

Sparrow and Wright (1974) isolated AMD hypersensitive mutants and studied their effect on cuticle development. The seven strains isolated were dominant lethals on food containing AMD and recessive lethals on standard food. Addition of AMD to the food at any time caused death at the following molt and this time could not be advanced by increasing the level of AMD, showing that AMD was an inhibitor of cuticular development. All these mutants mapped to a single locus, 53.1±0.5, designated as *l(2) amd*. There was no appreciable difference in the DDC activity levels of newly eclosed adults of these mutant heterozygotes compared with their control strains. Thus, while AMD could be used to discriminate strains with increased levels of DDC, it could not distinguish strains with decreased DDC levels.

A location of the structural gene for DDC was proposed by Hodgetts (1975). Using the methodology developed by Lindsley *et al.* (1972) of segmental aneuploidy to test the genome of *Drosophila* for DDC-dosage sensitive regions, he found that DDC activity was affected by the duplication of only one region in the genome--36EF-37D on the left arm of the second chromosome. Deletions and duplications in this region were associated with approximately 50% decreases and 50% increases in the enzyme activity as compared to euploids containing two copies of this region. The *amd* locus was also seen to be located in the DDC-dosage sensitive region 36EF-37D (Wright *et al.*, 1976a). Organisms with different doses of this region were differentially sensitive to AMD in food, strains containing a single dose being more sensitive than those containing two copies of this region and strains with three copies were more resistant to AMD than euploids.

Several deficiencies that delete the DDC-dosage sensitive region and the *amd* locus were isolated and studied by Wright *et al.* (1976a). The deficiencies were carried as heterozygotes over a balancer chromosome and the DDC activities of these heterozygotes were measured. Four deficiencies, *Df 50*, *Df 130*, *Df 158*, and *Df E71* showed approximately 50% of the activity of controls, indicating that they carry only one copy of the DDC-dosage sensitive region. Cytogenetic data showed that these deficiencies overlap and that the region 37B10-37C7 was deleted by all four deficiencies. The DDC-dosage sensitive region must be located in this region. The *amd* locus was also located in this region firstly because no *amd* / *Df* segregants from an appropriate cross involving these four deficiencies could be obtained. Secondly, the four deficiency heterozygotes were more sensitive to AMD in their food than control strains.

The authors suggested that the dosage sensitivity of the region 37B10-37C7 indicated the presence of the structural gene for DDC in this region. There could be other possibilities as observed by Rawls and Fristrom (1974) who, in addition to expected structural gene dosage responses, found flies hyperploid for several autosomal regions displayed altered levels of isocitrate dehydrogenase and α-glycerophosphate dehydrogenase. O'Brien and Gethman (1973) have pointed out that dosage sensitivity can only indicate the probable presence of a structural gene locus but cannot confirm this fact.

Wright *et al.* (1976b) isolated 26 recessive lethal mutations within *Df 130*, one of the deletions which included the putative structural gene for DDC. Amongst these lethals, eight DDC-deficient mutants were identified on the basis of the reduced DDC activity found in strains heterozygous for these mutations. The complementation analysis indicated that all eight mutations were allelic to each other and they were designated as *l(2) Ddc*^{n1} through *l(2) Ddc*^{n8}. The data presented in Table I summarize the properties of the two classes of mutants. All *Ddc* alleles complement with all *amd* alleles and therefore seem to belong to different genes. The *amd* locus mapped to the left of *Ddc* and the distance between the *Ddc* and *amd* loci, located in the 11 band region, 37B10-37C7, was calculated. Two pairs of alleles, *amd*^{H1} and *Ddc*^{n1} were less than 0.003 map units apart with a maximum estimated recombination frequency of 0.01% and *amd*^{H121} and *Ddc*^{n5} were approximately 0.0036 map units apart with a maximum recombination frequency of 0.017%.

The fact that the *amd* locus maps so close to the DDC structural gene locus suggests that they could be two groups of complementary mutations within the same cistron. However, DDC extracted from *amd*

TABLE I Summary of the characteristics of the DDC mutants and the AMD hypersensitive mutants

	DDC mutants	AMD hypersensitive mutants
i)	Recessive lethals	Recessive lethals
ii)	Map in the region 37B10-37C7	Map in the region 37B10-37C7
iii)	Homozygotes die at the end of embryogenesis; they are unable to hatch from the embryonic membranes. They do not exhibit any abnormalities of the anal organs.	Homozygotes die at the end of the embryonic period and show cuticular abnormalites on the last abdominal segment, lateral to the anus or across the ventral surface of the segment.
iv)	Heterozygotes are not hypersensitive to AMD in the food.	Heterozygotes are hypersensitive to AMD in the food.
v)	Heterozygotes show 28-53% of the control DDC activity.	Heterozygotes show approximately the same amount of DDC activity as the control stocks.
vi)	Ddc^{nx}/Ddc^{ny} progeny except Ddc^{n5}/Ddc^{n8} exhibit an "escaper" phenotype characterized by prolonged developmental time and die within 24 hours of eclosion, absence of normal pigmentation, presence of thin, long and straw-colored bristles, wing axillae and leg joints melanized and the flies walk on their tibia rather than tarsi but have coordinated movements.	*Hx/Hy* progeny do not exhibit the "escaper" phenotype.

vii) All DDC mutants complement with all AMD hypersensitive mutants.

viii) The *Ddc* locus maps to right of *amd*, approximately 0.003 map units apart.

strains was no different from their controls suggesting that these mutants have nothing to do with DDC. Further, *Ddc*^{n} / *CyO* heterozygotes were not affected by AMD in the food. Finally, the observed distance between *amd* and *Ddc* alleles is consistent with these being mutations in separate, probably adjacent genes. Wright *et al.* (1976b) suggested that the *Ddc* locus codes for the catalytic subunit of DDC while the *amd* locus could code for a separate DDC subunit carrying an allosteric site. However, Clark *et al.* (in press) have shown that DDC is in fact a dimer consisting of two subunits with the same molecular weight (54,000 daltons), and we feel it is unlikely that the allosteric subunit and catalytic subunit have the same molecular weight. While this is by no means excluded, we feel the additional data presented in this thesis, argues strongly against this possibility(see Discussion).

Another hypothesis to be considered is that the *amd* locus codes for an enzyme which binds to a substrate whose structure is similar to dopa. It need not be DDC but could be very closely related to DDC. Clark *et al.* (in press) postulated that the *Ddc* and *amd* genes could be divergent products of an ancient duplication event and that the enzyme which decarboxylates 5'-hydroxytryptophan could be the *l(2) amd* product. This is an area which has yet to be investigated.

Rawls and Fristrom (1975) believe that the *rudimentary* locus in *Drosophila* contains the structural gene of the first three enzymes in the pyrimidine biosynthetic pathway, *viz* carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase. Falk (1976) suggested that if not all three, at least the first two enzymes were contained within a single multifunctional protein complex. An understanding of

the need for the presence of multiple cistrons within a locus might lead to the understanding of gene regulation and the genome structure in higher eukaryotes. The *amd* and *Ddc* loci might represent one of these cases of multiple cistrons within a locus and, if pursued further, might reveal more interesting phenomena.

This project was undertaken to make use of the availability of highly purified DDC to prepare specific antibodies with which to probe the relationship between these two classes of mutations. The DDC activity levels and the amount of immunological cross-reacting material in the two classes were investigated and the data were interpreted in the light of the known subunit structure of DDC (P. Pass, personal communication).

A second goal of this work was to use the antibody to screen for naturally occurring wild type strains exhibiting excessive amounts of DDC. Chovnick *et al.* (1976) found a stock associated with more than normal levels of xanthine dehydrogenase (XDH) activity and this character mapped near the *rosy* locus which is the structural gene for XDH. Presumably there was no alteration in the structure of the XDH polypeptide since kinetic and immunological experiments indicated that higher levels of XDH in this stock were due to an increase in number of XDH molecules/fly. Fine structure recombination experiments showed that the genetic site for this character causing variation in number of molecules of XDH/fly, was very close to, but definitely outside of the XDH structural gene. Chovnick *et al.* (1976) present evidence against the over production of XDH being due to tandem duplication of the XDH structural element. The genetic element located adjacent to

the XDH structural gene appears to function as a cis-acting regulator of the XDH structural gene. We are interested in the possibility that similar controlling elements may lie adjacent to the DDC gene.

MATERIALS AND METHODS

Drosophila melanogaster stocks

Cultures of *Drosophila melanogaster* were reared at 25±1°C in half pint milk bottles in a standard medium consisting of 10% yeast, 10% sucrose, 1.5% agar, 1% propionic acid, and 0.01% chloramphenicol (Nash and Bell, 1968). Canton-Special (C.S.) was used as the wild type strain. A number of wild type stocks isogenic for a different second chromosome and designated as Bloomington Indiana (BI)-1, BI-3, BI-11 were obtained from Dr. Glenn Bewley. The descriptions of all the genetic markers and balancer chromosomes mentioned here are given in Lindsley and Grell (1968).

The DDC mutants and the hypersensitive mutants were obtained from Dr. T.R.F. Wright's laboratory. All of these are EMS induced recessive lethal mutants. The DDC mutants were isolated on the basis of their effect on DDC activity (Wright *et al.*, 1976b) and AMD hypersensitive mutants on the basis of their sensitivity to the substrate analogue AMD (Sparrow and Wright, 1974). The DDC mutants and the AMD hypersensitive mutants were maintained as heterozygotes in a balanced lethal system (see Table II). The control stocks, C-9 (Table II) for *Ddc* mutants and *HC8* (Table II) for *amd* mutants carry unidentified second chromosome recessive lethals and were recovered from the screen after mutagenesis. The control for *Ddc* mutants did not have reduced DDC activity and the control for *amd* mutants was not hypersensitive to AMD in the food.

Df (2L) 130 cn bw/CyO, a 12 band deficiency covering the *Ddc* and *amd* loci (Wright *et al.*, 1976a), and *Dp G1/X, dp b H121 pr/CyO*,

a duplication including this region (R.B.Hodgetts, personal communication) were used as controls.

All the *Drosophila melanogaster* stocks used with the exception of the isogenic stocks are given in Table II.

Chemicals

DDC assay: Oxifluor, Aquasol-2 and radioactive compounds (New England Nuclear).

DDC purification: Polyethylene glycol-6000 (Matheson, Coleman and Bell); alumina C_γ (Sigma); DE32 (DEAE, microgranular, Whatman Biochemicals Ltd.); Sephadex G-200 (Pharmacia) and hydroxyapatite (HAP, Bio-Gel HTP, Bio-Rad Laboratories).

Acrylamide gel electrophoresis: Acrylamide (Eastman Chemicals or Terochem Laboratories) recrystallized from chloroform; N, N-methylene-bisacrylamide (bisacrylamide, Eastman Chemicals) recrystallized from acetone, N, N, N', N'-tetramethylethylenediamine (TEMED Eastman Chemicals) redistilled *in vacuo*.

Protein determination: Bovine serum albumin (Sigma).

Immunoelectrophoresis: Agarose (L'Industrie Biologique Francaise S.A.).

Dopa Decarboxylase Assay

The enzyme DDC was assayed by the radiometric $^{14}CO_2$ microdiffusion method of Lunan and Mitchell (1969) as modified by Clark *et al.*(in press). The $^{14}CO_2$ produced by the decarboxylation of DL dopa 1-^{14}C using a 20 minute reaction period was trapped in oxifluor and counted by liquid scintillation.

Table II *Drosophila melanogaster* stocks used

Abbreviated stock designation	Genotypic description	References
C.S.	wild type	Lindsley and Grell (1968)
control-9 (C-9)	*rdo hk pr l(2)/CyO*[a]	Wright *et al.* (1976b)
Ddc^{n1}/CyO	*rdo hk Ddc^{n1} pr/CyO*	"
Ddc^{n2}/CyO	*rdo hk Ddc^{n2} pr/CyO*	"
Ddc^{n3}/CyO	*rdo hk Ddc^{n3} pr/CyO*	"
Ddc^{n4}/CyO	*rdo hk Ddc^{n4} pr/CyO*	"
Ddc^{n5}/CyO	*rdo hk Ddc^{n5} pr/CyO*	"
Ddc^{n6}/CyO	*rdo hk Ddc^{n6} pr/CyO*	"
Ddc^{n7}/CyO	*rdo hk Ddc^{n7} pr/CyO*	"
Ddc^{n8}/CyO	*rdo hk Ddc^{n8} pr/CyO*	"
Ddc^{n5}/Ddc^{n8}	*rdo hk Ddc^{n5} pr/rdo hk Ddc^{n8} pr*	constructed by the author
Df 130/CyO	*Df (2L) 130 cn bw/CyO*	Wright *et al.* (1976a)
Dp G1	*Dp(2;Y) G1/$\hat{X}Y$, y/$\hat{X}Y$, y; dp b l(2) amd^{H121} pr/CyO*[c]	R.B. Hodgetts, pers. comm.
control-8 (*HC8*)	*HC8 cn bw l(2)/SM5*[b]	Sparrow and Wright (1974)
H1/SM5	*l(2) amd^{H1} cn bw/SM5*	"
H45/SM5	*l(2) amd^{H45} cn bw/SM5*	"
H82/SM5	*l(2) amd^{H82} cn bw/SM5*	"
H121/SM5	*l(2) amd^{H121} cn bw/SM5*	"

a *CyO = In (2LR) O, dp^{lvI} Cy pr cn^2*

b *SM5 = In (2LR) SM5, al^2 Cy lt^v cn^2 sp^2*

c *Dp G1* ♂s bear the duplication, the females are euploids and are used as controls

DDC purification

DDC was purified from C.S. third instar larvae and white prepupae using the procedure of Clark *et al.* (in press). Approximately 200 gm of larvae were collected from plastic trays containing standard medium and live yeast. These trays were left in a population cage containing several thousand adult flies for a day and then incubated at 25°C until the mature third instar larvae crawled up the sides of the tray. The larvae were collected in water, washed several times, dried, weighed, and stored at -40°C or used immediatedly.

Protein determination

Protein was determined by the procedure of Lowry *et al.* (1951) using bovine serum albumin as the standard. The samples were dialyzed extensively against distilled water prior to determination. For small samples this procedure was modified by decreasing the sample and reagent volumes by a factor of 4.

Protein in column eluates was monitored by measurement of the absorbance at 280 mn.

Acrylamide gel electrophoresis.

All gels were made as described by Hedrick and Smith (1968) with the addition of 0.062% TEMED. The Tris-asparagine reservoir buffer system of these workers was used. The separating gels were 8.5% acrylamide (w/v). The column eluates were concentrated and then brought to 10% glycerol (v/v) before loading.

Vertical slab gel electrophoresis: The slab gel apparatus used was similar to the one described in Studier (1973). The gels were run at

6 mA for 1 hour and then at 10 mA for 3 hours. After electrophoresis the gels were removed from the glass plates and stained overnight in 0.05% Coomassie Blue (w/v) in 25% isopropyl alcohol (v/v) and 10% acetic acid (v/v). They were destained either by diffusion in 7% acetic acid or by transverse electrophoresis in solvent.

Disc gel electrophoresis: The gels were cast in glass cylinders with an internal diameter of 0.6 cm and a length of 10 cm. The separating gels were 7.5 cm long and the stacking gels were about 0.7 cm in length. The cylinders were then placed in a disc gel apparatus (Buchler Instruments) and the gels were run for 3½ - 4 hours at 1.5 mA (constant current)/tube. The gels were removed, stained and destained as mentioned above.

Gel scans were done with a Joyce-Loebl Chromoscan.

Preparation of rabbit anti-DDC serum

Four male San Juan Island rabbits were immunized against *Drosophila melanogaster* DDC; two against partially purified DDC and the other two against pure DDC. Rabbits were always bled either through the middle ear artery or the peripheral ear vein. Control serum from each rabbit was obtained not more than a week in advance of immunization.

Blood was allowed to clot for 2 hours at room temperature; the clot was seperated from the sides of the tube which was then left overnight at 4°C to allow the serum to seep out of the clot. On the next day the fluid was poured into another tube without disturbing the clot and centrifuged at 1085 x g for 20 minutes. The supernatant was centrifuged again if it was not clear and this was repeated until

a clear serum was obtained. The serum was stored in 1 ml aliquots at -40°C.

The antiserum obtained after immunizing the rabbits against DDC purified through to the DEAE step will hereafter be referred to as antiserum A. A concentrated sample of 2.8 ml of DDC (Table III, Purification #1) was added in 3 parts to an equal volume of Freund's complete adjuvant (Difco Laboratories). The mixture was homogenized in a Virtis homogenizer between each addition of the enzyme until a homogeneous emulsion was obtained. One drop of 1% ethyl mercurithiosalicylate (sodium salt) was added to the emulsion. This antigen was divided into two equal parts and injected subcutaneously between the shoulder blades of two rabbits.

Both rabbits were given a booster shot four weeks later with an antigen mixture containing 1.5 ml of DDC (Table III,Purification #2) and Freund's incomplete adjuvant instead of complete adjuvant. The rabbits were then bled one or two weeks after the booster shot and the antiserum A obtained was frozen in aliquots at -40°C.

The antiserum obtained after immunizing rabbits against pure DDC will be referred to as antiserum B. The same procedure as mentioned above was used for immunization, 1.5 ml of DDC sample (Table III, Purification #3) was taken to obtain the antigen for the first injection. A booster shot was given with 1.5 ml of DDC sample (Table III, Purification #4).

Prior to use, the immunoglobulins were partially purified by a 45% ammonium sulfate fractionation and the pellets were resuspended in an equal volume of 0.05 M Tris-HCl, pH 7.2 at 22°C.

Qualitative immunological analysis

The double immunodiffusion technique developed by Ouchterlony (1958) was used to detect the presence of rabbit anti-DDC antibodies. IDF-I cells from Cordis Laboratories were used. DDC samples were added to the outer wells and antiserum to the centre well and the cells were incubated at 18±1°C for 60 hours or until the precipitin lines were clearly visible.

Quantitative precipitin analysis

A double incubation technique was used to measure the amount of cross-reacting material (CRM) present in the crude extracts from various strains. The extracts were made in 0.05 M Tris-HCl, pH 7.2 at 22°C, containing 1 mM phenylthiourea (PTU) and contained 20 mg flies (equal numbers of 0-2 hour old male and female flies) per ml. Experimental extract (125 µl) was incubated at 4°C for 3 hours with an equal volume of crude immunoglobulin preparation obtained from antiserum A (immunoglobulin I) diluted 1:50 in 0.05 M Tris-HCl. These samples were centrifuged at 34,800 x g for 10 minutes. The supernatant (100 µl) was then incubated with an equal volume of standard crude extract from C.S. flies for 3 hours. The amount of antibody remaining was determined by the percentage of the enzyme activity remaining in the standard crude extract and is a measure of the CRM level in the experimental strain. The amount of immunoglobulin I used in the first incubation was only just slightly more than required to inactivate a standard crude extract with 100% CRM.

Quantitative immunoelectrophoresis

A quantitative procedure for the estimation of CRM as described by Laurell (1965, 1966) was also used. Different amounts of an antigen produce "rocket" shaped precipitin lines when electrophoresed into an agarose gel of uniform thickness containing homogeneously distributed antibody to that antigen. The height of the rocket was used as a measure of the CRM levels.

The slab gel dimensions were 13 X 16 X 0.3 cm. A final concentration of 1% (w/v) agarose, in Gelman Tris-Barbital electrophoresis buffer (pH 8.8) at an ionic strength of 0.05, was obtained by heating the mixture in a boiling water bath. The solution was cooled to 45±1°C and 62.5 µl of crude immunoglobulin preparation obtained from antiserum B (immunoglobulin II) was added. This agarose-antibody mixture was then poured into the gel mold described by Studier (1973). The gels were allowed to set for 30 minutes after which the clamps were removed and the upper glass plate was slipped off carefully. A row of 12 holes 1.1 cm apart, parallel to the long edge and 2 cm from it, was cut with a #2 cork borer (5 mm diameter). The gel was placed horizontally in a Gelman electrophoresis apparatus containing the electrophoresis buffer in both chambers. The gel was connected to the chambers on either side by filter papers soaked in buffer. The samples were then applied to the holes and the gels were run at 130 volts (constant voltage) for 6 hours at 4°C. After the completion of electrophoresis the gels were incubated in 250 ml of 0.2 M NaCl overnight and stained for 30 minutes in 0.05% (w/v) Coomassie Blue in 45.4% (v/v) methanol and 9.2% (v/v) acetic acid. Later they were destained by diffusion in 25% (v/v) methanol and 7.5% (v/v) acetic

acid.

Samples for analysis were obtained after partial purification of a crude extract containing 200 mg of flies (equal numbers of 0-2 hour old males and females) per ml in 0.05 M Tris-HCl, pH 7.2 at 22°C, containing 1 mM PTU. The proteins precipitated after a 53-73% saturated $(NH_4)_2SO_4$ fractionation were resuspended in half the initial volume in 0.05 M Tris-HCl, pH 7.2 at 22°C. Different amounts of these samples: 5, 10, and 15 µl were loaded into the wells and electrophoresis was carried out. Single rockets were observed above each well in the stained gels. These were measured to the nearest 0.25 mm from the leading edge of the well to the tip of the rocket. Linear regression lines and the estimated slopes were computed from the observed values. CRM was calculated as the ratio of the slopes of the experimental to the control strain. A standard error of the ratio was also calculated.

RESULTS

DDC purification

DDC was purified according to the procedure of Clark *et al.* (in press). Four separate preparations were made. In the first two, 200 gm of freshly frozen third instar larvae were ground in a mortar with a pestle to obtain the crude extract and the purification was carried through to the completion of the chromatography on DEAE. For the next two preparations, 200 gm of freshly frozen third instar larvae were homogenized in a Waring blender to make the crude extract; these preparations were carried through the entire purification procedure.

The amount of protein and DDC activity in each crude and final extract were determined. The specific activity and degree of purification of each preparation were calculated and are summarized in Table III. Polyacrylamide gel electrophoresis was performed on concentrated samples and the results are shown in Fig. 1a,b. While several bands were observed after electrophoresis of the material purified through DEAE chromatography (Fig. 1a), a single band was observed in the preparation carried through the complete purification scheme (Fig. 1b).

A densitometer scan was done on the gel in Fig. 1b and a single prominent peak was observed. This peak corresponded to the DDC activity peak as shown in Fig. 2.

Characterization of antisera A and B

Antisera obtained were characterized by the double diffusion technique developed by Ouchterlony (1958). Antiserum A obtained after

Table III Summary of DDC purification data

purification step	final volume (in ml)	µg protein/ml	activity in cpm /50 µl	specific activity cpm/mg protein	fold purification
Preparation #1					
crude extract	740	15670	11,000	1.4×10	-
DEAE concentrate	4.5	264	55,000	4.17×10^3	297
Preparation #2					
crude extract	910	13790	12,700	1.8×10	-
DEAE concentrate	4	231	57,000	4.93×10^3	268
Preparation #3					
crude extract	1004	49000	11,900	$.48 \times 10$	-
HAP concentrate	2	205	48,000	4.68×10^3	964
Preparation #4					
crude extract	1050	42000	12,500	$.59 \times 10$	-
HAP concentrate	3	154	45,500	5.91×10^3	993

Figure 1 Acrylamide gel electrophoresis of samples taken at different stages in the procedure for purifying DDC

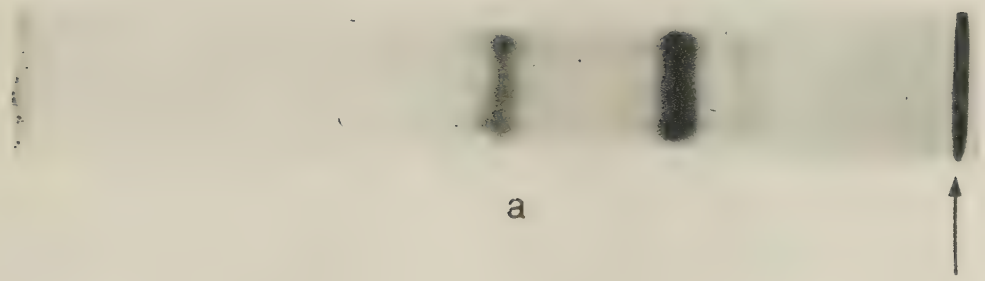
a) Slab gel electrophoresis of the eluate from the DEAE column

8.5% acrylamide gel was loaded with 26 µg of concentrated eluate (Preparation #1, Table III) and run at 6 mA for 1 hour and then at 10 mA for 3 hours. The gel was stained overnight in Coomassie Blue as explained in the Materials and Methods.

b) Disc gel electrophoresis of the eluate from the HAP column

An 8.5% acrylamide gel was loaded with 20 µg of concentrated eluate (Preparation #3, Table III) and run at 1.5 mA/tube for 4 hours. The gels were stained overnight in Coomassie Blue as explained in the Materials and Methods.

The arrows mark the tracking dye.

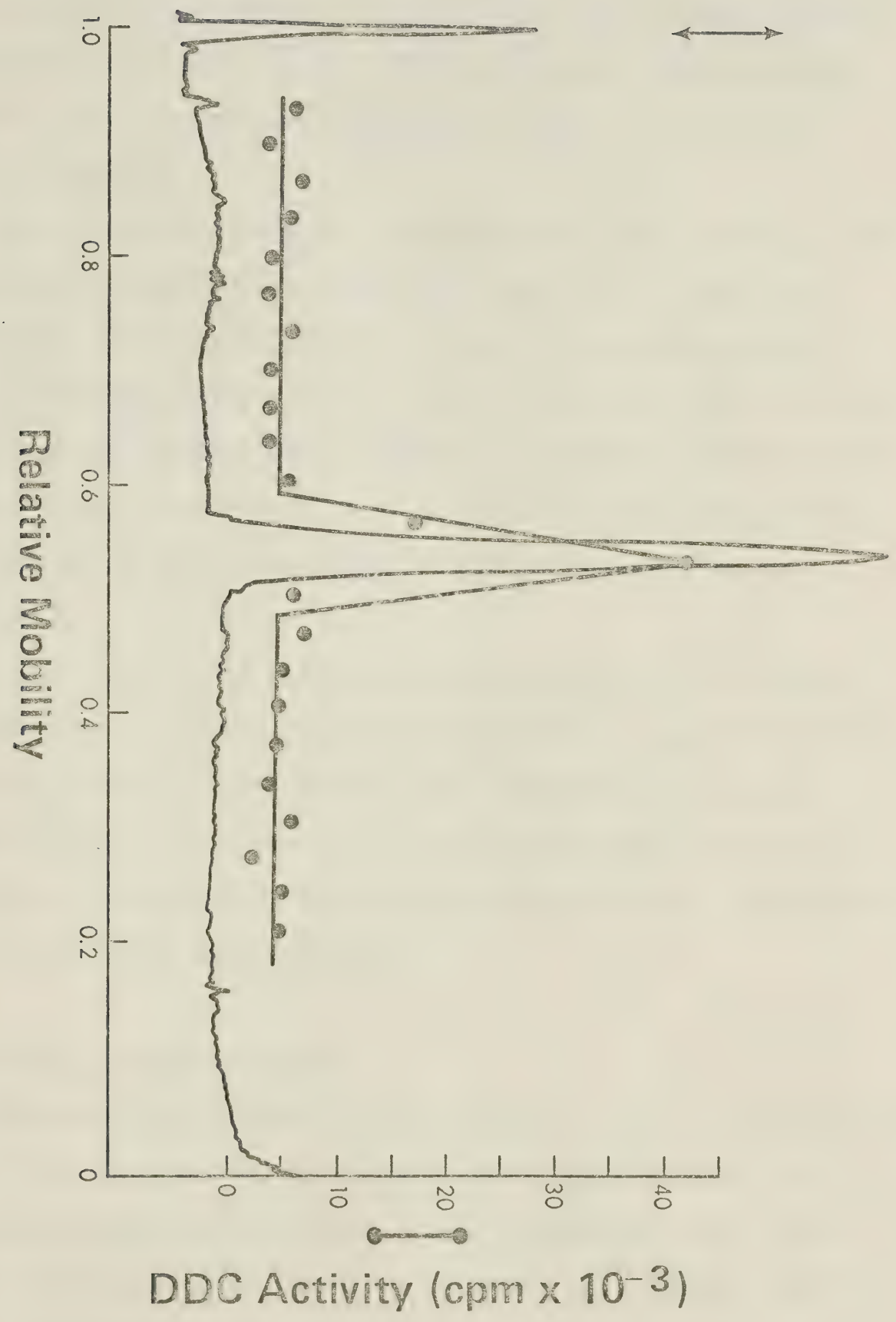


a

b

Figure 2 Microdensitometer scan and DDC activity measurements of disc gels of eluate from HAP column

The microdensitometer scan of a disc gel loaded with 20 µg of concentrated eluate (Preparation #3, Table III) shows a single peak. A gel which was run parallel to the first one was sliced into 2 mm slices and assayed for DDC activity (William Clark, personal communication). The double headed arrow marks the tracking dye.


Relative Mobility
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
DDC Activity (cpm x 10⁻³)

immunizing the rabbits with impure DDC (Preparations #1 and #2, Table III) was tested with crude extracts (20 mg/ml) in a double immunodiffusion cell and more than one band (Fig. 3a) was observed. This indicated that the serum contained antibodies against some other *Drosophila* proteins beside DDC.

The antiserum obtained after immunizing the rabbits with pure DDC (Preparations #3 and #4, Table III) gave one band with larval and adult crude extracts and a single band with a concentrated eluate from a HAP column (Preparation #4, Table III, Fig. 3b). This indicated that the serum contained mainly antibodies against *Drosophila* DDC and no other *Drosophila* proteins. In Fig. 3b all the bands are connected and there are no spurs, suggesting that larval and adult DDC are identical.

The anti-DDC antiserum obtained against *Drosophila melanogaster* was tested for its ability to form antigen-antibody complexes with DDC from other insects. While the DDC from a *Calliphoran*, *Cynomyopsis cadaverina*, was not the same as the one from *Drosophila melanogaster* and was not precipitable by anti-*Drosophila* DDC antibodies, the DDC from *Drosophila simulans* was precipitable.

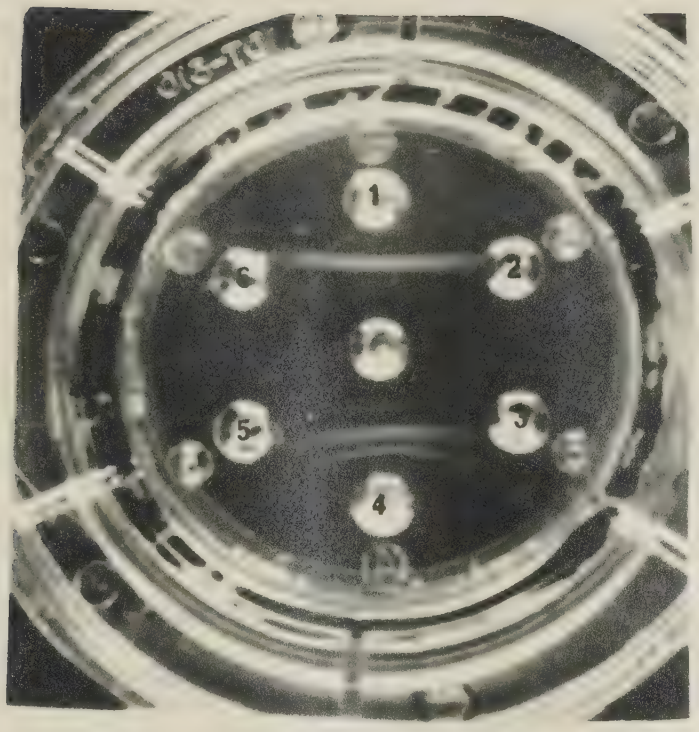
Quantitative precipitin analysis

Antiserum A was employed for these analyses, and the immunoglobulins in both the control and antisera were partially purified by a 45% saturated $(NH_4)_2SO_4$ precipitation. Prior to routine analysis, some preliminary experiments were done to determine the incubation time for complete antigen-antibody complex formation and the titer.

Figure 3 Qualitative immunological analysis of antisera A and B

a) Double diffusion cells with antiserum A elicited against impure DDC. The centre well contained the antiserum and well 1 contained C.S. adult crude extract (20 mg/ml) and well 4 contained C.S. larval crude extract (20 mg/ml).

b) Double diffusion cells with antiserum B elicited against pure DDC. The centre well contained the antiserum; wells 1 and 4 contained C.S. larval crude extract (20 mg/ml); wells 2 and 5 contained C.S. adult crude extract (20 mg/ml) and wells 3 and 6 contained HAP eluate (205 µg/ml).



a

b

Two experiments using different crude extracts were done to determine the optimum incubation time. Equal volumes of immunoglobulin I (1:50 dilution with 0.05 M Tris-HCl, pH 7.2 at 22°C) and crude extracts of C.S. flies were incubated for varying lengths of time between ½ hour and 5 hours at 4°C. The samples were centrifuged at 34,800 x g for 10 minutes and the supernatants were assayed for DDC activity. Fig. 4 shows the results obtained and from these data, 3 hours was chosen as the standard incubation time.

Three experiments using different crude extracts were done to determine the titer of immunoglobulin I. Equal volumes of diluted immunoglobulin A and crude extracts were incubated for 3 hours at 4°C after which the samples were centrifuged at 34,800 x g for 10 minutes. The % DDC activity inhibited by the antibodies in each case was calculated. Fig. 5a indicates that a dilution factor of 1:50 was optimum.

A confirmation of this result was obtained using a somewhat different protocol. To 100 µl of crude extract 25, 50, 75, 100, 125, 150 or 200 µl of immunoglobulin I (1:50 dilution) was added and the final volume was adjusted to 300 µl by addition of 0.05 M Tris-HCl, pH 7.2 at 22°C. Samples were incubated at 4°C, centrifuged at 34,800 x g for 10 minutes and the supernatants were assayed for DDC activity. The % activity inhibited was calculated and is shown in Fig. 5b.

After the determination of these parameters, quantitative precipitin analyses were performed as outlined in Materials and Methods. The linearity of the technique was evaluated by taking 25, 50, 75, and 100 µl samples of a crude extract of C.S. flies, incubating these with the

Figure 4 Determination of optimum incubation time for the formation of the antigen-antibody complex

Aliquots of crude extract from C.S. flies (20 mg/ml) were incubated with a 1:50 dilution of immunoglobulin I for different lengths of time. Samples were centrifuged and the supernatants were assayed for DDC activity.
Two experiments using two different crude extracts were done.

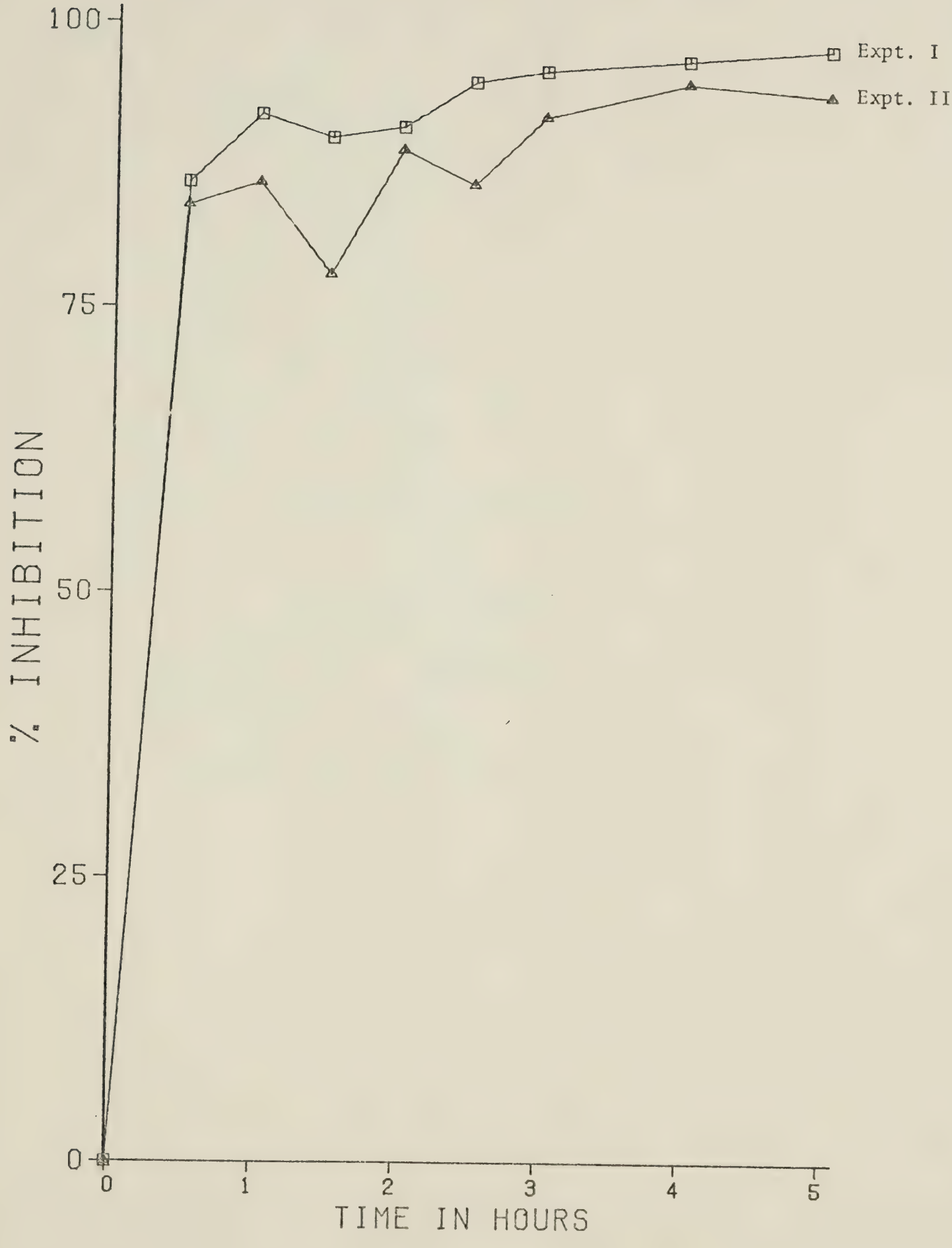

100
75
50
25
0
% INHIBITION
0
1
2
3
4
5
TIME IN HOURS
Expt. I
Expt. II

Figure 5a Titer determination of immunoglobulin I -- Protocol I

Crude extracts from C.S. flies (20 mg/ml) were incubated for 3 hours at 4°C with different dilutions of immunoglobulin I. Equal volumes of extract and diluted immunoglobulin A were used. The mixtures were centrifuged and the supernatants were assayed for DDC activity. Three different experiments were done using different crude extracts.

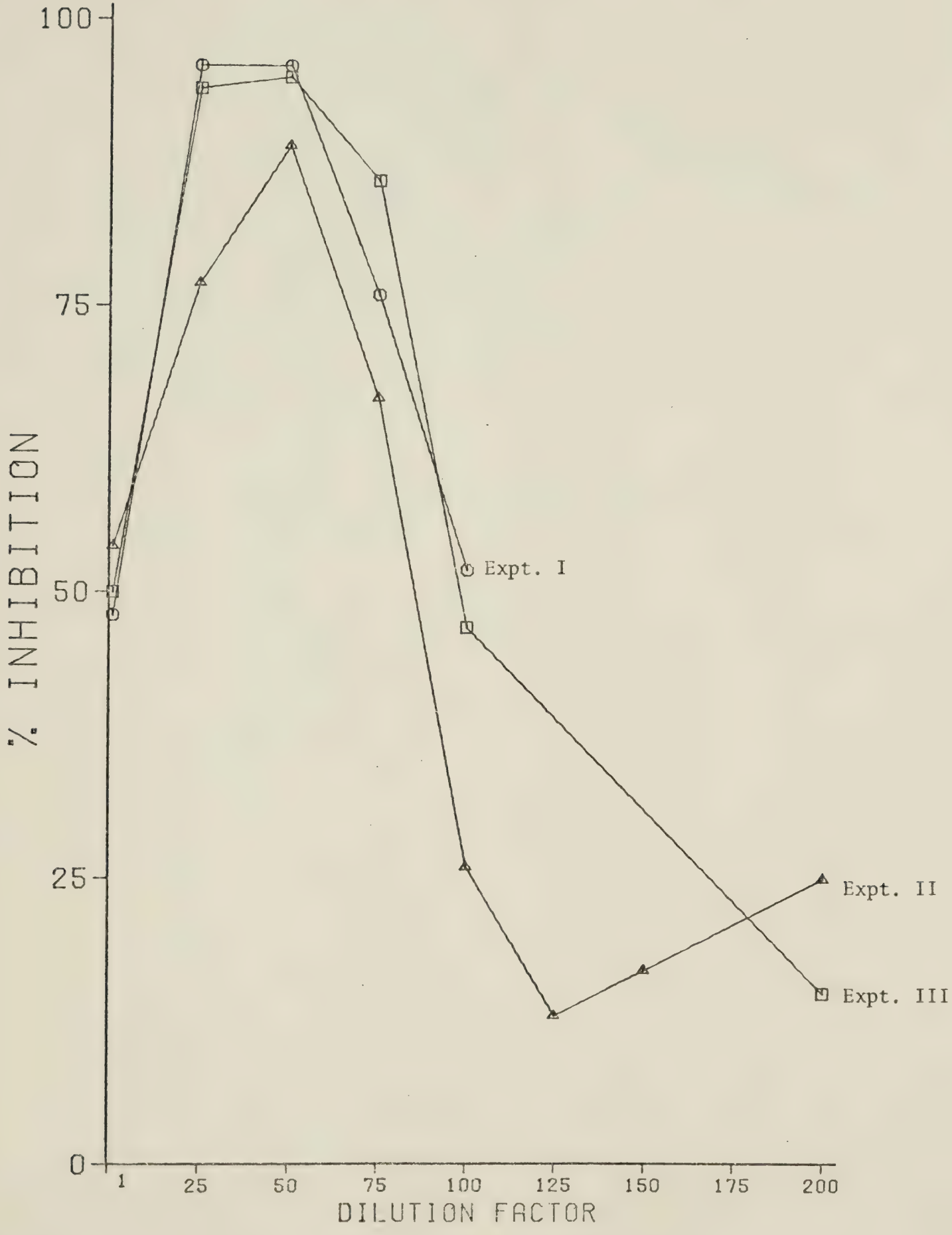
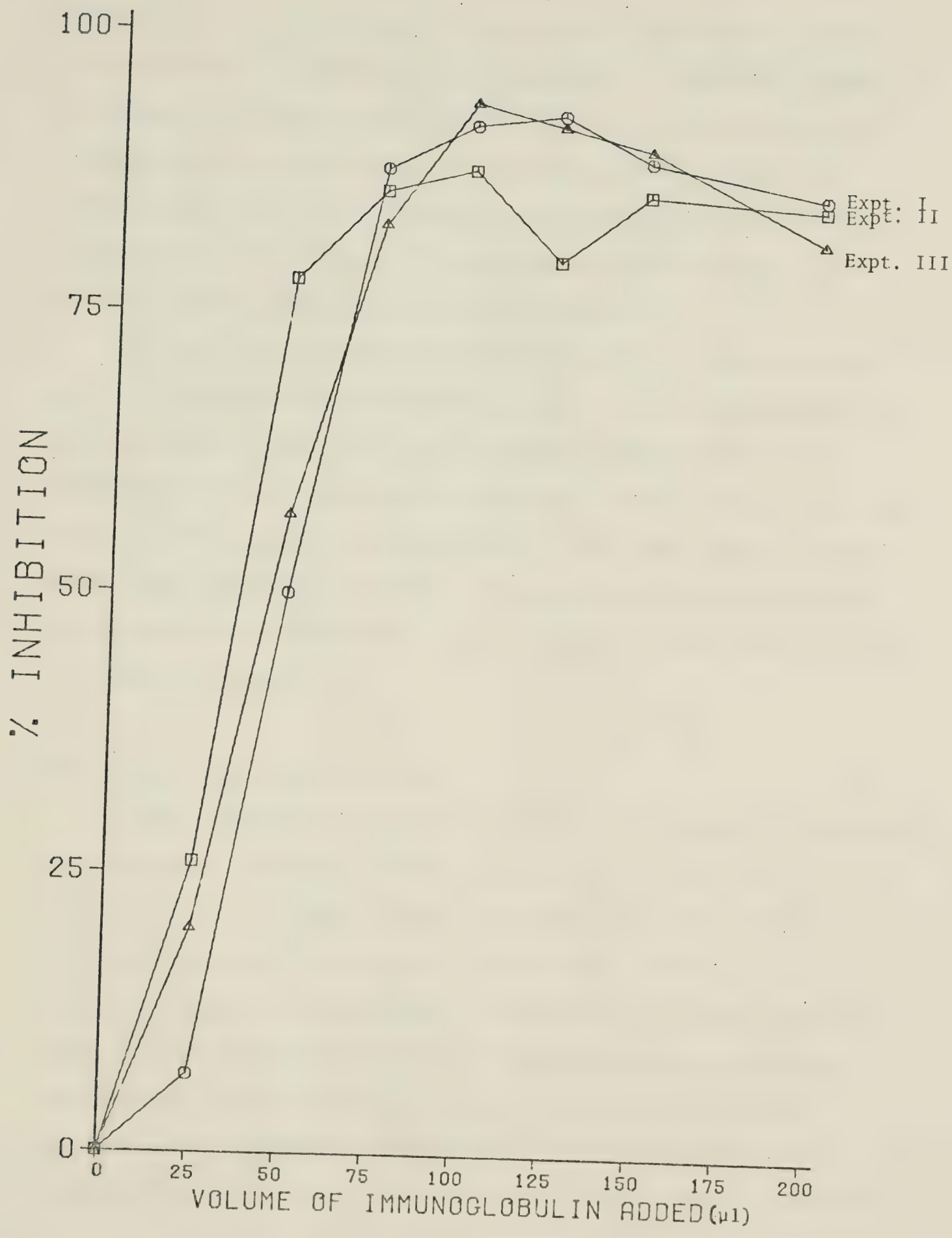

100
75
50
25
0
% INHIBITION
1
25
50
75
100
125
150
175
200
DILUTION FACTOR
Expt. I
Expt. II
Expt. III

Figure 5b Titer determination of immunoglobulin I -- Protocol II

A fixed volume of crude extract from C.S. flies (20 mg/ml) was incubated for 3 hours at 4°C with different volumes of 1:50 diluted immunoglobulin I which had been partially purified. The samples were centrifuged and the supernatants were assayed for DDC activity.

Three separate experiments were done.


100
75
50
25
0
% INHIBITION
0
25
50
75
100
125
150
175
200
VOLUME OF IMMUNOGLOBULIN ADDED (µl)
Expt. I
Expt. II
Expt. III

immunoglobulin I and assaying the supernatants of the second incubation for DDC activity to calculate the % CRM present. The values obtained are summarized in Table IV and the means and standard deviations were calculated based on the assumption that each value was independent. Table IV shows that the technique gives a reasonably accurate measure of the amount of CRM present. The % CRM observed in different mutant strains by this technique is summarized in Table V.

To justify the expression of CRM as a function of live weight and not as a function of the total protein present in the crude extracts, the protein levels in three different *Ddc* mutants (each belonging to a different class, see Discussion), their control C-9 and the C.S. flies were determined. The amount of protein present in the crude extracts of these strains are summarized in Table VI. There was no appreciable difference in the amount of protein present in the different mutants, their control, or in the C.S. strain.

Quantitative immunoelectrophoresis

In this technique, as the antigen migrates into the gel it encounters the antibodies and antigen-antibody complexes are formed; these precipitate to give "rocket" shaped precipitin lines. Since the antibodies are evenly distributed in the gel, the heights of the rockets observed were proportional to the amount of antigen loaded; the heights of the rockets increased with increasing amounts of antigen. The linearity of this technique was tested by using 0.1-0.7 µg of DDC (102 µg/ml, Table III, Preparation #3) and observing the rocket heights (Fig. 6). The plot of rocket heights against amount of DDC (Fig. 7)

Table IV Evaluation of the linearity of the quantitative precipitin technique

µl of crude extract of C S flies	µl of Tris-HCl buffer	% CRM expected	% CRM observed				mean	standard deviation
			Expt. 1		Expt. 2			
			a	b	a	b		
25	75	25	25.66	20.85	26.37	24.09	24	2
50	50	50	41.22	44.64	46.22	49.44	45	3
75	25	75	74.66	76.65	70.94	74.62	74	2
100	0	100	100	100	100	100	-	-

Table V % CRM obtained by quantitative precipitin analysis

Strain	% CRM observed[a]				mean	standard deviation
	Expt. 1		Expt. 2			
	a	b	a	b		
Duplication	143.85	146.46	141.46	134.69	141	5
Deficiency	45.16	49.61	47.29	44.51	47	2
Ddc^{n1}/CyO	39.79	44.79	34.55	42.71	40	4
Ddc^{n2}/CyO	54.50	47.58	49.25	45.16	49	4
Ddc^{n3}/CyO	51.04	53.63	47.63	52.14	51	4
Ddc^{n4}/CyO	40.57	43.94	38.85	44.76	42	3
Ddc^{n5}/CyO	77.68	73.01	77.37	73.28	75	3
Ddc^{n6}/CyO	37.87	40.92	32.71	37.82	37	3
Ddc^{n7}/CyO	48.10	44.81	46.00	50.71	47	3
Ddc^{n8}/CyO	76.40	79.76	83.64	78.10	79	3
Ddc^{n5}/Ddc^{n8}	12.65	14.23	19.25	16.55	16	3
H1/SM5	90.69	93.61	97.41	93.45	94	3
H45/SM5	91.64	94.96	92.24	96.13	94	2
H82/SM5	96.88	94.24	90.84	98.34	95	3
H121/SM5	90.95	94.24	87.21	92.84	91	3

[a]CRM is expressed as % of control. The C-9 strain was used as the control for all *Ddc* mutants, *Ddc^{n5}/Ddc^{n8}*, and the deficiency bearing strain. The *HC8* strain was the control for the *amd* mutants. Euploid females of the stock *Dp G1* were used as controls for the *Dp G1* ♂s bearing the duplication of the DDC-dosage sensitive region.

Table VI Amount of protein present in crude extracts of different mutants and the C.S. strain

Strain	amount of protein[a] µg protein / ml
C.S.	1155
C-9	1250
Ddc^{n5}/*CyO*	1275
Ddc^{n6}/*CyO*	1225
Ddc^{n7}/*CyO*	1200

[a]Protein was estimated in crude extracts containing 20 mg flies/ml

Figure 6 Standard curve: Quantitative immunoelectrophoresis

Wells # 1-7 of a 1% agarose gel containing antibody were loaded with 1-7 µl of HAP concentrate (102 µg protein/ml) and the gel was run for 6 hours at 130 volts at 4°C. The gel was incubated overnight in 0.2 M NaCl and stained in Coomassie Blue.

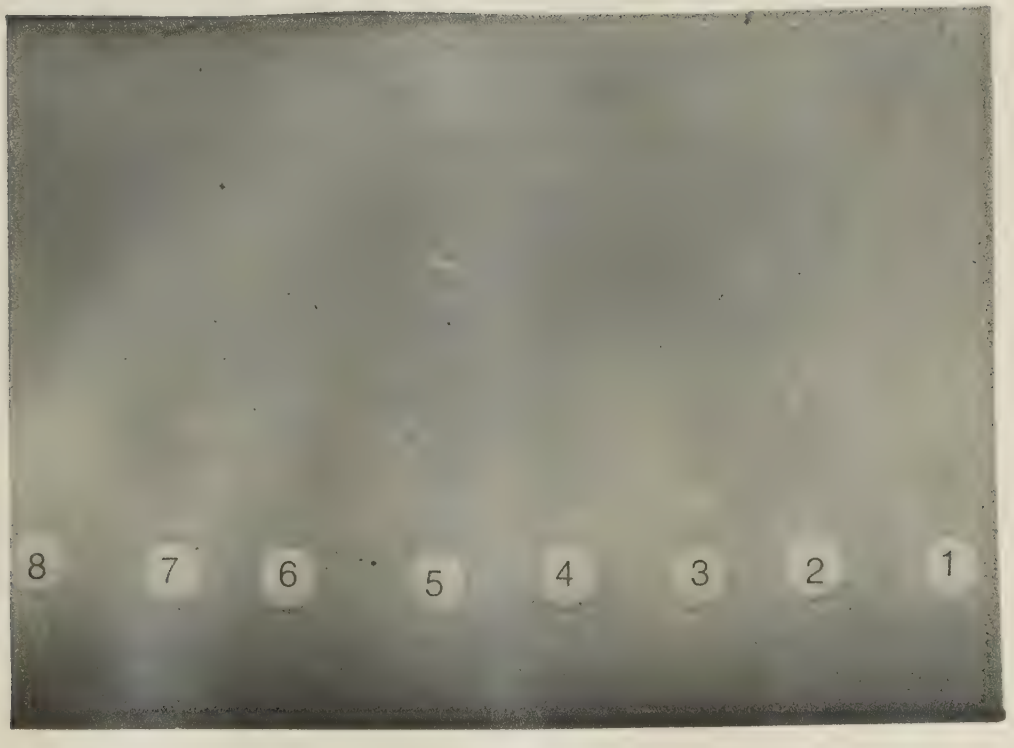
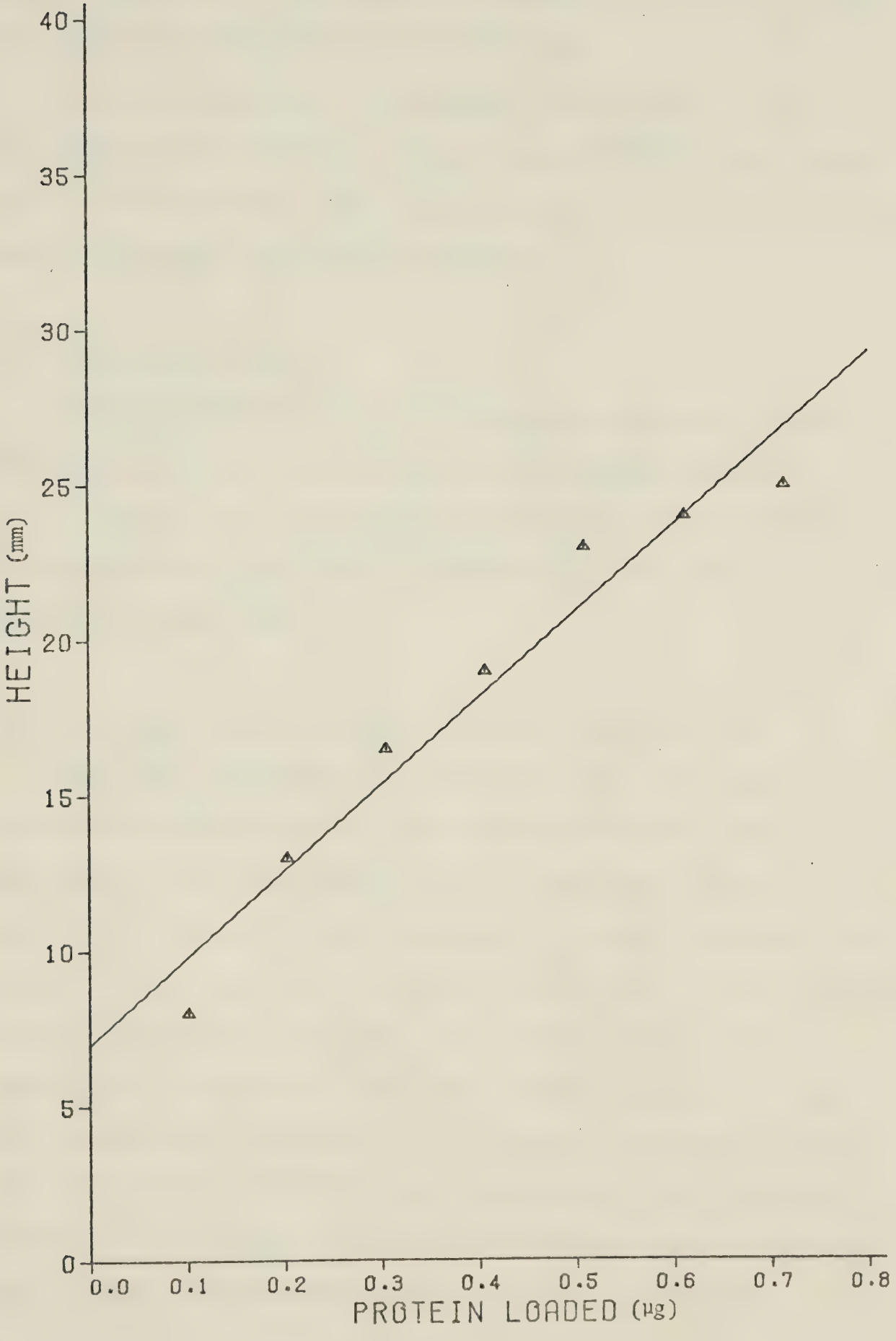

8
7
6
5
4
3
2
1

Figure 7 Standard curve: Quantitative immunoelectrophoresis

Various volumes of DDC purified to homogeneity and containing 102 µg protein/ml were loaded onto a 1% agarose gel containing antibody. The gel was run for 6 hours at 130 volts at 4°C, incubated in 0.2 M NaCl overnight, and stained in Coomassie Blue for ½ hour. The rockets observed were measured to the nearest 0.25 mm.

The linear regression line and the slope of the line were computed and the correlation coefficient was also calculated. The correlation coefficient was 0.975.


HEIGHT (mm)
40
35
30
25
20
15
10
5
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
PROTEIN LOADED (µg)

appears linear up to 20 mm and 0.5 µg of protein. All points obtained later for CRM estimates fell within this range.

The results obtained for the mutants are presented in Figs. 8-11 and are summarized in Table VII. Samples from Ddc^{n5}/Ddc^{n8} flies did not produce distinct rockets over the wells and hence the CRM level for this double heterozygote could not be determined.

DDC activity measurements

DDC activity in the different strains was assayed in the same crude extract as used for the quantitative precipitin analysis. The values obtained, their means and the standard deviations calculated on the assumption that each value was independent of the others, are summarized in Table VIII.

Screen for altered levels of DDC activity in wild type stocks

Twenty-seven wild type stocks including C.S., each isogenic for a different second chromosome, were screened for altered levels of DDC activity. The crude extracts for the assay were prepared in the same way as those used for the quantitative precipitin analysis. The assays were done in triplicate and the average counts for the different stocks are presented in Table IX. The activity of each stock was expressed as a percentage of the overall average. The results were also expressed as a percentage of our standard laboratory wild type stock. Since stocks BI-78, BI-133, BI-140, BI-155, BI-3, and BI-164 appeared to differ significantly from the average, each was reassayed. However only the strain BI-140 gave a repeatable result with a

Figure 8 Quantitation of CRM in a duplication bearing genotype by immunoelectrophoresis

For each genotype, duplication bearing males and euploid control females, a crude extract containing 200 mg flies/ml was concentrated two-fold by a 53-73% saturated $(NH_4)_2SO_4$ fractionation in 0.05 M Tris-HCl, pH 7.2 at 22°C. A 1% agarose gel containing antibody was loaded with 5, 10, and 15 µl of the samples and run at 130 volts for 6 hours at 4°C, incubated in 0.2 M NaCl overnight, and stained in Coomassie Blue as described in the Materials and Methods. The rockets observed were measured to the nearest 0.25 mm and the linear regression lines plotted; the slopes were calculated and tabulated in Table VII.

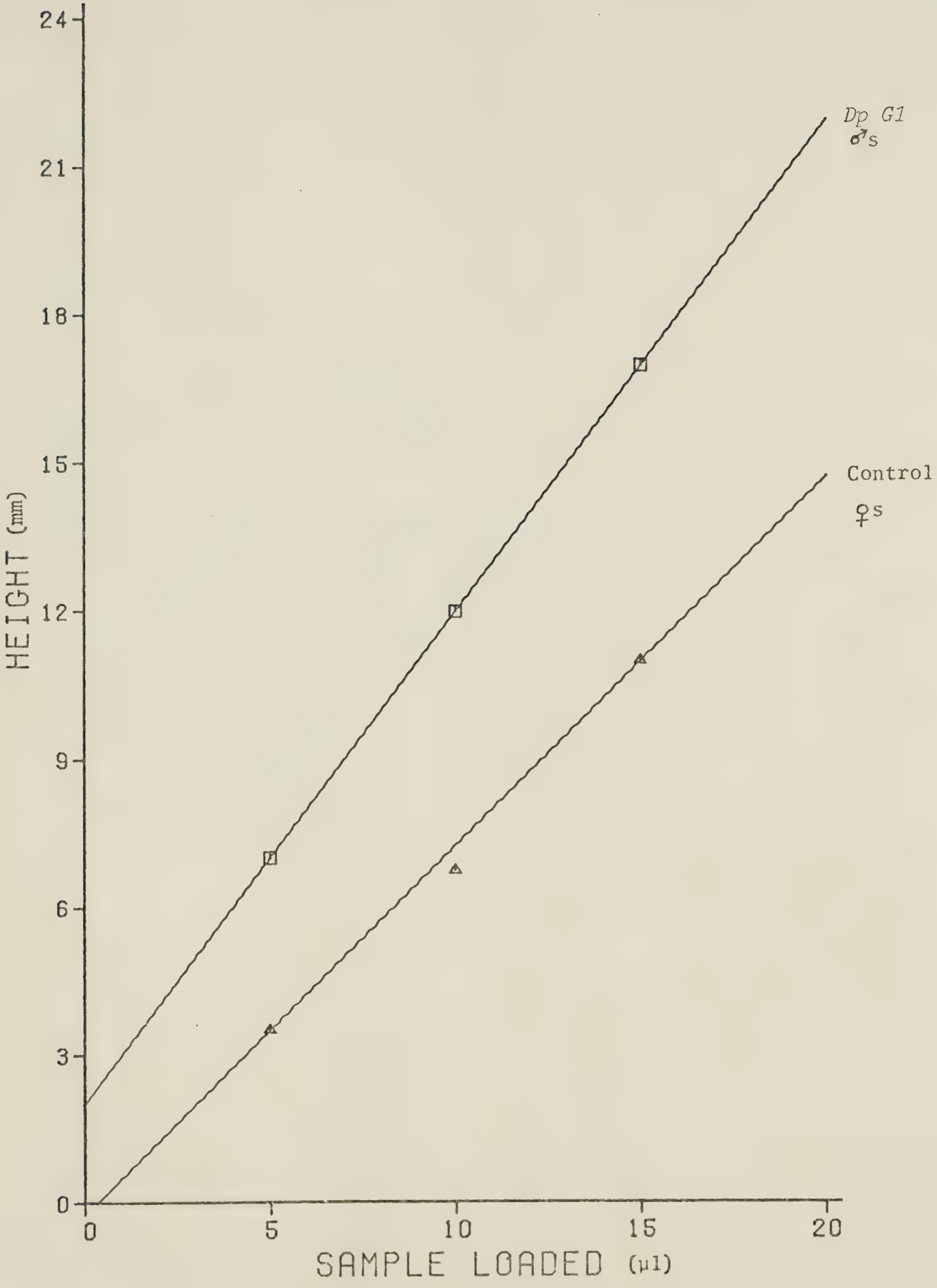

24
21
18
15
12
9
6
3
0
HEIGHT (mm)
0
5
10
15
20
SAMPLE LOADED (µl)
Dp G1
♂s
Control
♀s

Figure 9 Quantitation of CRM in a deficiency bearing genotype by immunoelectrophoresis

For each genotype, *Df 130/CyO* and its control C-9, a crude extract containing 200 mg flies/ml was concentrated two-fold by a 53-73% $(NH_4)_2SO_4$ fractionation in 0.05 M Tris-HCl, pH 7.2 at 22°C. A 1% agarose gel containing antibody was loaded with 5, 10, and 15 µl of the samples and run at 130 volts for 6 hours at 4°C, incubated in 0.2 M NaCl overnight, and stained in Coomassie Blue as described in the Materials and Methods. The rockets observed were measured to the nearest 0.25 mm and the linear regression lines plotted; the slopes were calculated and tabulated in Table VII.

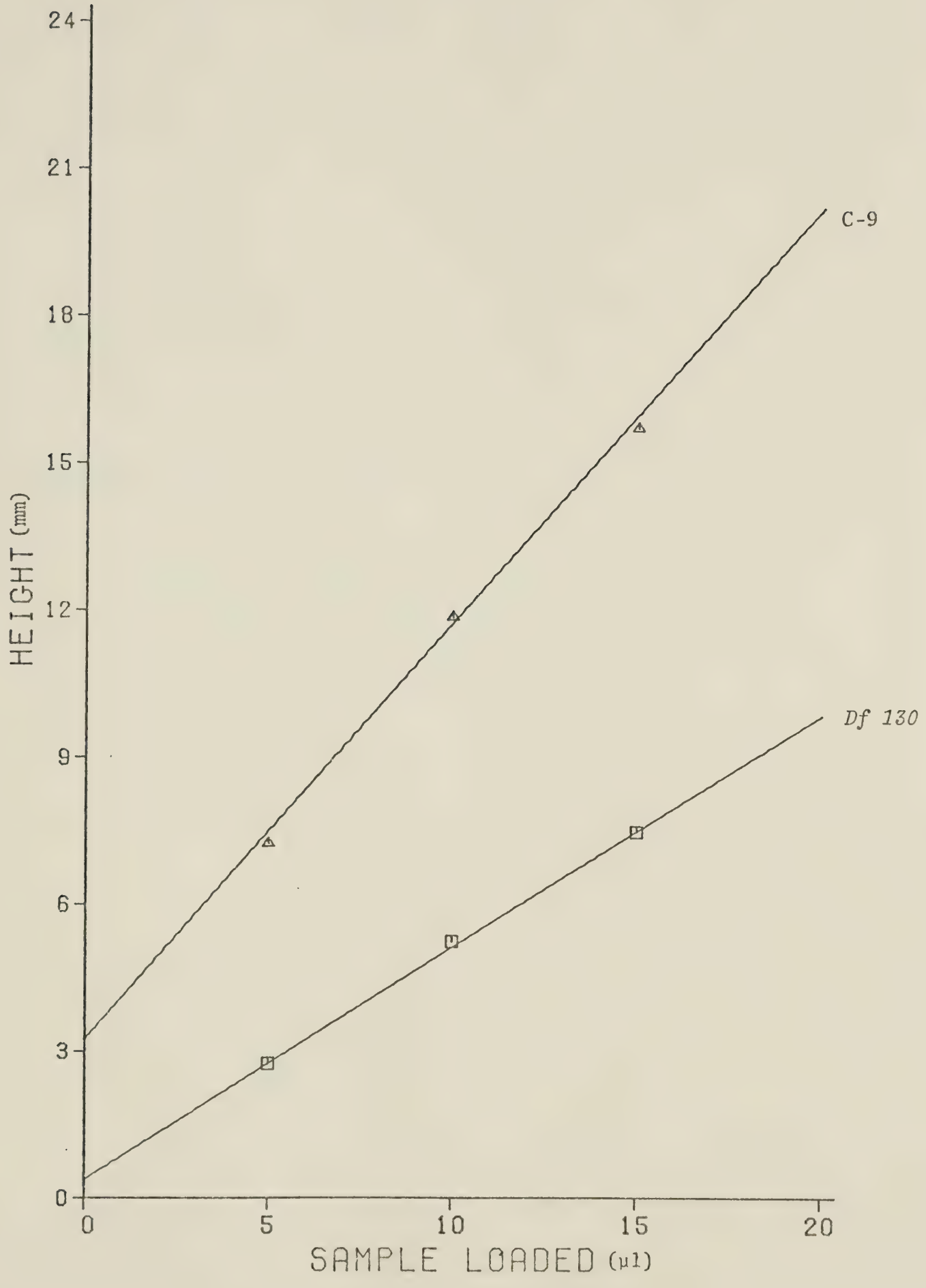

24
21
18
15
12
9
6
3
0
HEIGHT (mm)
0
5
10
15
20
SAMPLE LOADED (µl)
C-9
Df 130

Figure 10 Quantitation of CRM in *Ddc* mutants by immunoelectrophoresis

For each genotype, all *Ddc* mutants and their control C-9, a crude extract containing 200 mg flies/ml was concentrated two-fold by a 53-73% saturated $(NH_4)_2SO_4$ fractionation in 0.05 M Tris-HCl, pH 7.2 at 22°C. A 1% agarose gel containing antibody was loaded with 5, 10, and 15 µl of the samples and run for 6 hours at 4°C, incubated in 0.2 M NaCl overnight, and stained in Coomassie Blue as described in the Materials and Methods. The rockets observed were measured to the nearest 0.25 mm and the linear regression lines plotted; the slopes were calculated and tabulated in Table VII.

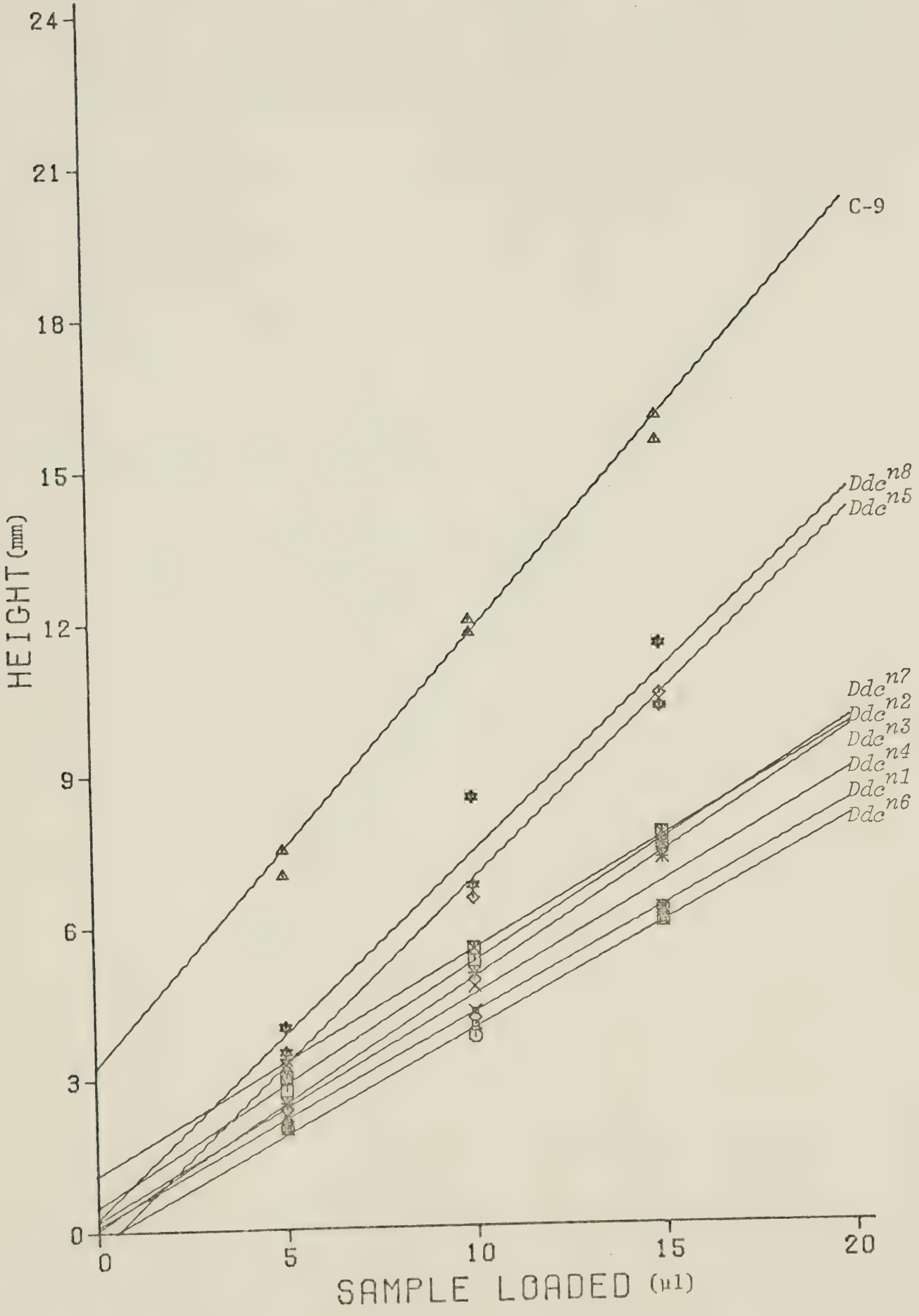

24
21
18
15
12
9
6
3
0
HEIGHT (mm)
0
5
10
15
20
SAMPLE LOADED (µl)
C-9
Ddc n8
Ddc n5
Ddc n7
Ddc n2
Ddc n3
Ddc n4
Ddc n1
Ddc n6

Figure 11 Quantitation of CRM in *amd* mutants by immunoelectrophoresis

For each genotype, all *amd* mutants and their control *HC8*, a crude extract containing 200 mg flies/ml was concentrated two-fold by a 53-73% saturated $(NH_4)_2SO_4$ fractionation in 0.05 M Tris-HCl, pH 7.2 at 22°C. A 1% agarose gel containing antibody was loaded with 5, 10, and 15 µl of the samples and run at 130 volts for 6 hours at 4°C, incubated in 0.2 M NaCl overnight, and stained in Coomassie Blue as described in the Materials and Methods. The rockets observed were measured to the nearest 0.25 mm and the linear regression lines plotted; the slopes were calculated and tabulated in Table VII.

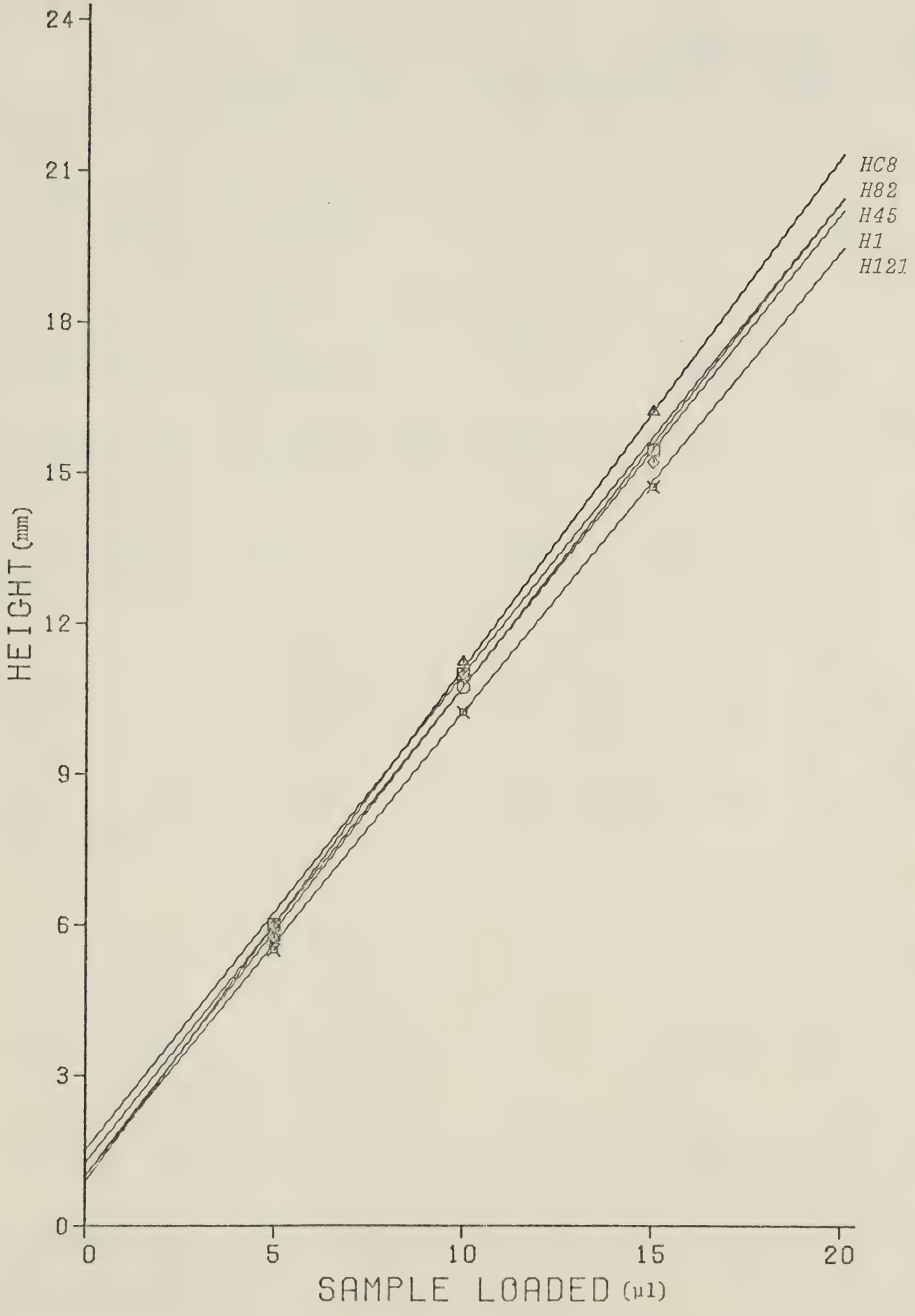

24
21
18
15
12
9
6
3
0
HEIGHT (mm)
0
5
10
15
20
SAMPLE LOADED (µl)
HC8
H82
H45
H1
H121

Table VII. % CRM obtained by quantitative immunoelectrophoresis

	height in mm						estimate of slope b	ratio[a] be/bc % CRM	standard error
	Expt. 1			Expt. 2					
µl of sample	5	10	15	5	10	15			
Strain									
control	3.50	6.75	11.00				0.7500		
Dp G1	7.00	12.00	17.00				1.0000	133	10
C-9	7.00	12.00	16.00	7.50	11.75	15.50	0.8500		
Df 130	2.75	5.25	7.50				0.4750	56	3
Ddc^{n1}/CyO	2.00	3.75	6.25	2.00	3.75	6.00	0.4125	49	3
Ddc^{n2}/CyO	2.75	5.25	7.50	3.00	5.50	7.75	0.4750	56	3
Ddc^{n3}/CyO	3.25	5.50	7.75	3.25	5.50	7.50	0.4375	51	2
Ddc^{n4}/CyO	2.50	4.75	7.25	2.25	4.25	6.25	0.4375	51	5
Ddc^{n5}/CyO	3.00	6.50	10.25	3.25	6.50	10.50	0.7250	85	4
Ddc^{n6}/CyO	2.25	4.25	6.50	2.00	4.00	6.00	0.4125	49	3
Ddc^{n7}/CyO	2.50	5.00	7.50	2.50	5.00	7.25	0.4875	57	3
Ddc^{n8}/CyO	4.00	8.50	11.50	3.50	6.75	10.25	0.7125	84	10
Ddc^{n5}/Ddc^{n8}	[b]								
HC8	6.00	11.25	16.25				1.0250		
H1/SM5	6.00	11.00	15.50				0.9500	93	3
H45/SM5	5.75	11.00	15.50				0.9500	93	6
H82/SM5	5.75	10.75	15.50				0.9750	95	2
H121/SM5	5.50	10.25	14.75				0.9250	90	2

[a] CRM is expressed as the ratio of the slope of the experimental strain (be) to that of the appropriate control strain (bc). See Legend Table VIII.

[b] No rocket shaped precipitin lines were observed.

Table VIII DDC activity measurements

Strain	% DDC activity observed[a] Expt. 1 a	Expt. 1 b	Expt. 2 a	Expt. 2 b	mean	standard deviation
Duplication	142.75	145.69	153.26	151.42	148	5
Deficiency	47.49	51.76	54.71	60.18	54	5
Ddc^{n1}/CyO	26.21	28.95	27.28	29.85	28	2
Ddc^{n2}/CyO	43.57	46.31	47.06	44.09	45	2
Ddc^{n3}/CyO	47.23	48.40	46.05	47.58	47	1
Ddc^{n4}/CyO	24.63	28.27	29.06	30.49	28	3
Ddc^{n5}/CyO	24.52	32.31	30.96	34.67	31	4
Ddc^{n6}/CyO	28.42	32.59	28.48	23.53	28	4
Ddc^{n7}/CyO	46.79	51.14	59.15	61.92	55	7
Ddc^{n8}/CyO	33.27	36.22	35.29	40.24	36	3
Ddc^{n5}/Ddc^{n8}	5.46	6.02	8.67	10.22	8	2
H1/SM5	92.43	89.61	83.21	87.21	88	4
H45/SM5	86.36	92.00	80.66	84.78	86	5
H82/SM5	92.99	87.35	84.17	82.11	87	5
H121/SM5	85.64	82.84	81.15	76.31	82	4

[a] CRM is expressed as % of control. The C-9 strains was used as the control for all *Ddc* mutants, *Ddc^{n5}/Ddc^{n8}*, and the deficiency bearing strain. The *HC8* strain was the control for the *amd* mutants. Euploid females of the stock *DpG1* were used as controls for the *Dp G1* ♂s bearing the duplication of the DDC-dosage sensitive region.

Table IX Screening stocks for altered levels of DDC activity

Stock	Average activity (in cpm per 50 µl)	% activity (of average)	% activity (of C.S.)
C.S.	5828	109	100
BI-3	4840	91	83
BI-4	6260	116	107
BI-15	4661	87	80
BI-27	5590	104	96
BI-30	5880	109	101
BI-32	5116	95	88
BI-37	4900	91	84
BI-46	5190	97	89
BI-54	4470	83	77
BI-62	6310	117	108
BI-76	5520	103	95
BI-78	6750	125	114
BI-90	6480	120	111
BI-99	5206	97	89
BI-133	6899	128	118
BI-134	5037	94	86
BI-139	4573	85	78
BI-140	7206	134	124
BI-144	5839	109	100
BI-148	4540	85	78
BI-149	4432	83	76
BI-155	4288	80	74
BI-156	4348	81	75
BI-157	5578	104	96
BI-162	5658	105	97
BI-164	3928	73	68

higher level of DDC activity. BI-140 had 24 % more activity than did C.S. and its CRM level was estimated by quantitative precipitin analysis; the results are presented in Table X.

Table X DDC activity and CRM in strain BI-140

Stock	% activity (of C.S.)[a]	% CRM (of C.S.)[a]
BI-140	124 ± 2	120 ± 5

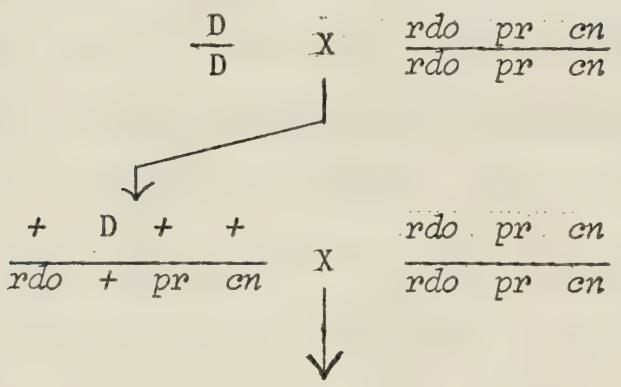
[a]Activity and CRM measurements were done on separate crude extracts and two measurements of each were obtained.

DISCUSSION

Mutations in both structural genes and the regulatory elements controlling these genes could affect the level of enzyme activity in any organism. In this thesis we have studied a number of mutants affecting the level of the enzyme DDC. Several of these mutants appear to be in the structural gene for DDC and one mutant could be a putative regulatory mutant of the enzyme.

The presence of regulatory loci is known both in mice and *Drosophila*. The best studied example is the regulatory site just adjacent to the XDH structural gene in *Drosophila* (Chovnick *et al.* 1976). In an attempt to identify putative regulatory loci for DDC, we screened 26 naturally occurring strains of *Drosophila melanogaster*, each isogenic for a different second chromosome. These were kindly provided by Dr. Glenn Bewley. Of the 26 stocks screened for altered levels of DDC activity, only one BI 140, consistently demonstrated abnormal activity. The 24% increased activity that we found was apparently due to an overproduction of DDC, since the strain had 20% more CRM than normal. Chovnick *et al.* (1976) observed that the character which causes an over production of XDH maps adjacent to the XDH structural gene. It causes a 400% increase in both XDH activity and CRM and acts as a cis- regulator. The strain BI 140 which shows increased levels of both DDC activity and CRM could contain an analogous mutation affecting the regulation of DDC. This has to be pursued further genetically. The following mapping scheme (Fig. 12) could be used to map a character D, which we postulate causes an increase in the amount of DDC. We are assuming that D maps between *rdo* and *pr*, as does the *Ddc* gene.

Figure 12 Mapping scheme for the character controlling increased levels of DDC activity



Select for *rdo* + and + *pr* recombinants over the *rdo pr cn* chromosome, assuming that D maps between *rdo* and *pr*.

rdo + + / *rdo pr cn* X *rdo pr cn* / *CyO*

Select for *Cy* non *pr* progeny.

+ *pr* + / *rdo pr cn* X *rdo pr cn* / *CyO*

Select for *Cy pr* progeny.

rdo + / *CyO* X *rdo* + / *CyO*

Select for non *Cy* progeny.

+ *pr* / *CyO* X + *pr* / *CyO*

Select for non *Cy* progeny.

rdo + / *rdo* +

+ *pr* / + *pr*

CyO = *In (2LR) O, dp*^{lvI} *Cy pr cn*^{2}

The scheme allows for the production of stocks homozygous for reciprocal recombinant chromosomes *rdo +* and *+ pr*. If this character maps close to *rdo*, then the recombinants *rdo +* made into homozygous strain would not exhibit increased level of DDC activity, where as the recombinants *+ pr* as a homozygous strain would show increased level of DDC activity. If it mapped close to *pr* the reverse would be observed. If this character does map close to the *Ddc* gene between *rdo* and *pr*, further studies to determine whether it acts as a cis-dominant controlling element would be of interest. If D does not map in the *rdo-pr* region, it may turn out to be a locus affecting the DDC structural gene in a matter similar to that by which one of the loci *mal*, *cin* or *lxd* affects the activity of XDH. All three of these loci are removed from the *ry* locus which is the structural gene for XDH.

The antisera elicited against both partially purified and pure *Drosophila* DDC contained anti-DDC antibodies as shown by their ability to inactivate crude preparations of DDC. Antiserum A raised against partially purified DDC contained antibodies against more than one *Drosophila* protein (Fig. 3a) whereas antiserum B, which was raised against pure DDC was monospecific (Fig. 3b). Fig. 3b also shows that DDC from larvae and adults are identical as they form connected bands with no spurs in a double diffusion cell containing the anti-DDC antiserum. This was further confirmed by the complete precipitation of DDC from adult extracts with the anti-DDC antiserum elicited against the larval enzyme. This allowed us to use the antibody raised against larvae to monitor CRM levels in adults.

Antibody reaction with a soluble antigen could lead to precipitation or flocculation. In the first case, in the region of antibody excess, all antigen is complexed and precipitated. In the second case, the complexes

are soluble in excess antibody. Generally, rabbit antisera contains only precipitating antibody; the flocculating system is best seen with horse antisera. However, flocculation can occur under certain conditions with rabbit antisera also, and this may explain why we did not observe complete precipitation (i.e., 100% loss of enzyme activity in our supernatents with excess antibody].

The production of specific antibodies to an antigen depends on the structure of the antigen and for every antigen there exists a heterogeneous population of antibodies reacting with different determinants on the surface of the antigen. The antigen-antibody interactions causing the inhibition of enzyme activity could either be due to steric hindrance and/or conformational changes. The inhibition of enzyme activity is due mainly to steric effects which hinder the access of the substrate to the catalytic site (Michaelides *et al.*, 1964). However, antibodies also may cause conformational changes which inhibit enzyme activity (Najjar and Fisher, 1956) although the addition of specific substrates will protect the enzyme from subsequent inhibition by its antibodies (Samuels, 1963). We never observed 100% inhibition of DDC activity after precipitation with antibody (Fig. 4, 5a, and 5b). This may be due to partial solubility of a small fraction of the antigen-antibody complexes in which the enzyme is not completely inactivated.

Quantitative immunoelectrophoresis is a method which should measure the total antigen concentration, hopefully equally valid for enzymatically active and inactive forms of the protein. The immunoglobulins have to be homogeneously distributed throughout the antibody-containing gel to enable accurate quantitation by immunoelectrophoresis. In 'Rocket immunoelectrophoresis', the technique used in this study, the peak height and not the peak area is used to estimate the antigen content. The curve is usually

linear but deviations from linearity are observed when the antigen range is wide. In some cases, semilogarithmic plots of antigen concentrations against the peak heights give linear curves and this could be a result of the above mentioned deviation. The sensitivity of the technique differs slightly for different antigens and depends on the limit of visual detection of the 'rocket' shaped precipitin lines.

The DDC activity levels and CRM levels obtained by the two different techniques used in this thesis are summarized in Table XI. Even though quantitative immunoelectrophoresis gave a linear standard curve, the values observed for the strain carrying a deficiency in the DDC-dosage sensitive region and most of the *Ddc* mutants are higher than those observed by quantitative precipitin analysis. The values obtained by these two techniques probably cannot be averaged and hence are considered separately. Since the interpretation of our results is not altered in any substantive way by considering the data from either one of the two techniques, for convenience in the discussion we will consider only the data obtained by the quantitative precipitin analysis.

Syvanen and Roth (1973) observed that the gene coding for the regulatory subunit of aspartate transcarbamylase was closely linked and adjacent to the gene coding for the catalytic subunit of the same enzyme in *Salmonella typhimurium*. The results obtained from *Calliphora* (Fragoulis and Sekeris, 1975c) showed that the DDC from this insect was a heterodimer suggesting an enzyme structure similar to aspartate transcarbamylase. Preliminary results obtained in our laboratory supported the idea that DDC in *Drosophila melanogaster* was also a heterodimer. This led Wright *et al.* (1976b) to suggest that the *amd* gene which is closely linked to the *Ddc* gene, might be coding for a regulatory subunit of DDC while the *Ddc* gene codes for a catalytic subunit of DDC. A

Table XI Summary of CRM and activity levels of DDC in mutant strains

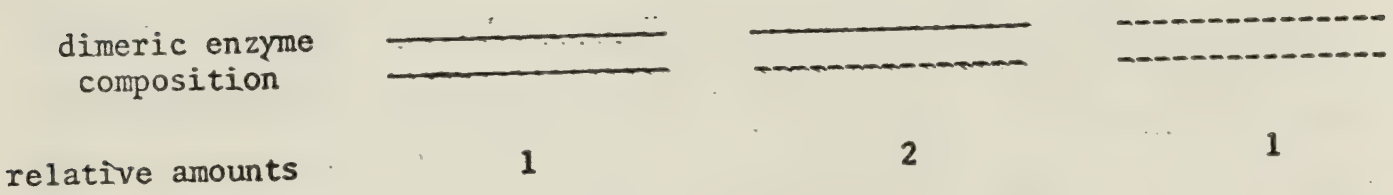
Strain	DDC CRM (% of control) Precipitin analysis mean and S.D.	DDC CRM (% of control) Immunoelectrophoresis mean and S.D.	DDC activity (% of control) mean and S.D.
Duplication	141 ± 5	133 ± 10	148 ± 5
Deficiency	47 ± 2	56 ± 3	54 ± 5
Class I			
Ddc^{n2}/CyO	49 ± 4	56 ± 3	45 ± 2
Ddc^{n3}/CyO	51 ± 4	51 ± 2	47 ± 1
Ddc^{n7}/CyO	47 ± 3	49 ± 3	55 ± 7
Class II			
Ddc^{n1}/CyO	49 ± 4	49 ± 3	28 ± 2
Ddc^{n4}/CyO	42 ± 3	51 ± 5	28 ± 3
Ddc^{n6}/CyO	37 ± 3	49 ± 3	28 ± 4
Class III			
Ddc^{n5}/CyO	75 ± 3	85 ± 4	31 ± 4
Ddc^{n8}/CyO	79 ± 3	84 ± 10	36 ± 3
Ddc^{n5}/Ddc^{n8}	16 ± 3		8 ± 2
H1/SM5	94 ± 3	93 ± 3	88 ± 4
H45/SM5	94 ± 2	93 ± 6	86 ± 5
H82/SM5	95 ± 3	95 ± 2	87 ± 5
H121/SM5	91 ± 3	90 ± 2	82 ± 4

number of observations now argue against this possibility. First, Clark *et al.* (in press) have shown by SDS electrophoresis that *Drosophila* DDC is a dimer consisting of two apparently identical subunits. Second, the DDC from *Drosophila* appears to be quite different from the DDC of *Calliphora*, since the DDC from *Calliphora* is not precipitable by anti-*Drosophila* DDC antibodies (see Results). Third, one might expect that if the *amd* gene coded for the regulatory subunit of DDC, mutants in this gene would affect the DDC activity or CRM levels. Table XI shows that mutants in the *amd* gene do not show any substantial changes in either the enzyme activity or the CRM level. Thus we favour the explanation advanced by Wright *et al.* (1976b) and elaborated by Clark *et al.* (in press) that *amd* and *Ddc* genes are the divergent products of an ancient duplication event and now code for related but different proteins. We postulate that dopa is decarboxylated primarily by DDC but also to a limited extent by the *amd* product, which retains some specificity for dopa. In the *amd* mutants, this product could either be absent or modified and hence not decarboxylate dopa. This could account for the consistently lower activity and CRM levels in the crude extracts of the *amd* mutants.

The CRM levels and the DDC activity measurements allow us to divide the *Ddc* mutants into three distinct classes. Mutants in class I, comprised of Ddc^{n2}, Ddc^{n3}, and Ddc^{n7}, had about 50% DDC activity and CRM. In class II, mutants Ddc^{n1}, Ddc^{n4}, and Ddc^{n6} had about 28% activity and 43% CRM of the control. In class III, mutants Ddc^{n5} and Ddc^{n8} had about 35% of the activity and 75% CRM of the control.

Knowing that the enzyme is a dimer (Clark *et al.*, in press) and assuming that these *Ddc* mutants are structural gene mutants and that only the dimers exhibit activity and CRM, the observations can be interpreted as follows (see Fig. 13): Class I mutants behave like complete null mutants--as heterozygotes they have 50% of the activity and CRM of their

Figure 13 Proposed model for DDC dimers in mutant heterozygotes



Class I — Only normal polypeptides associate to form normal dimers. Therefore, the heterozygotes have about 50% activity and CRM.

Class II

100% CRM^+	Partially CRM^+	not detected CRM^- and
100% activity	Little or no activity	inactive

The heterozygotes have about 25% activity and 43% CRM.

Class III

100% CRM^+	100% CRM^+	not detected CRM^- and
100% activity	Partially active	inactive

The heterozygotes have about 35% activity and 75% CRM.

———— normal polypeptide

---------- mutant polypeptide

control; the active enzyme which is CRM^+ is made from polypeptides from the *CyO* chromosome carrying the Ddc^+ allele. It appears as though the mutant polypeptides are not produced and if produced, do not interact with normal polypeptides. The class III mutant heterozygotes had 75% of the control CRM and 35% enzyme activity. The normal polypeptides probably associate to form CRM^+ dimers with 100% activity. The normal and mutant polypeptides probably associate to form a CRM^+ dimer with some activity. The class II mutant heterozygotes had 28% activity and about 43% CRM of the controls. The normal polypeptides probably associate to give CRM^+ active dimers. The mutant and normal polypeptides probably associate to give partially CRM^+ dimers which have little or no activity. The lower CRM levels observed in class II mutants compared to class III or class I mutants could be interpreted in one of two ways. First, the antigenicity of the mutant II/normal dimer is less than that of the mutant III/normal dimer (whose antigenicity equals that of normal/normal dimer). Or secondly, the mutant II/normal dimers are partially degraded.

The mutant III/normal dimer is as antigenic as the normal/normal dimer and this is consistent with the intracistronic complementation data for the *Ddc* mutants obtained by Wright *et al.* (1976b). The mutants Ddc^{n5} and Ddc^{n8} belonging to class III are the only two which complement enough to produce viable adults which do not exhibit the "escaper" phenotype (Table I). This suggests that the lesions in the *Ddc* gene in these two mutants are not severe and that these mutants produce polypeptides which are close to normal. The mutants belonging to other classes complement only slightly or not at all and rarely produce viable adults. The adults produced exhibit a severe "escaper" phenotype and die very early after eclosion. This suggests that the lesions in the *Ddc* gene in these mutants are severe and the mutant/normal dimer produced doesn't resemble the

normal/normal dimer. The mutants belonging to class I showed low levels of intracistronic complementation and Ddc^{n7} belonging to this class, does not complement with any of the other mutants. The CRM results and complementation data together suggest that mutants in class I appear to be complete null mutants.

The Ddc^{n5}/Ddc^{n8} flies had about 8% enzyme activity and 16% CRM. In these heteroallelic heterozygotes presumably the dimers mutant 5/ mutant 5 and mutant 8/ mutant 8 were CRM^- and inactive. The CRM and activity were contributed by the heteroallelic or hybrid dimers. The CRM level of this combination could not be obtained by quantitative immunoelectrophoresis because no distinct rockets were seen. There were streaks of stain all the way above the well after staining the gels, suggesting that the enzyme from these flies had reduced affinity for the antibodies against wild type DDC and may be structurally and/or antigenically different from the wild type enzyme.

The coupling of enzyme activity measurements with determinations of CRM has allowed us to examine the molecular basis of the mutant defects in the *Ddc* mutants in more detail than Wright *et al.* (1976b). In particular, the CRM studies have allowed us to clearly identify the class III mutants which on the basis of enzyme activity alone are not easily distinguishable from the class II mutants. The availability of highly specific antibody against DDC will allow us to persue other biochemical and genetic aspects in the study of DDC. For example, an antibody-affinity column prepared by P.Pass (personal communication) has greatly simplified our purification procedure for the enzyme. The antibody is being used in an attempt to develop a technique for visualizing DDC on gels following electrophoresis and, if successful, will permit the screening of electrophoretic variants

of the enzyme. And finally an attempt is being made to purify the mRNA for DDC by precipitation with antibody of specific polysomes in the process of translating DDC mRNA.

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